Schizosaccharomyces pombe Phosphatidylinositol 4-kinase, Pik1p, in cell cycle control

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Abstract

Pik1p, one of three phosphatidylinositol 4-kinases in the fission yeast, *Schizosaccharomyces pombe*, was found previously to interact with Cdc4p, a myosin essential light chain that is required for cytokinesis. The involvement of *pik1* in cell cycle control was investigated. A fluorescently tagged Pik1p fusion protein was associated with Golgi throughout the cycle, and was found at the medial division plane of the cell during late cytokinesis. This latter distribution has not been reported previously. Gene deletion in diploid cells and tetrad analysis revealed that *pik1* is essential for cell viability and is required for spore germination. The terminal phenotype of a temperature-sensitive, loss-of-function allele (*pik1-td*) indicated that *pik1* is involved in cytokinesis: particularly for suppression of secondary septum material deposition, for suppression of initiation of supernumerary septa, and for cell separation. Contractile ring formation was normal in *pik1-td* cells at the restrictive temperature although the pattern of F-actin patches was disrupted. The F-actin patches were dispersed throughout the cytoplasm. Accumulation of extra inner membranous or vesicle-like structures was observed in these cells. The *S. pombe nmt1* promoter and attenuated versions of it were found to be useful for complementation studies in *S. cerevisiae.* Heterologous expression of *S. pombe pik1* complemented the essential functions of a temperature-sensitive allele (*pik1-101*) of its orthologue in *Saccharomyces cerevisiae* that were lost at the restrictive temperature. A residue required for *S. pombe* Pik1p lipid kinase activity, D709, was also required for this complementation. A residue, R838, which is required for interactions between Pik1p and Cdc4p was not required for this complementation. The timing and localization of Pik1p to the division plane of the cell late in cytokinesis combined with analysis of the terminal phenotype of a loss-of-function allele, indicate that Pik1p and/or its derived phosphoinositides are required for regulation of septation and cell separation. Pik1p may be involved in the transport, possibly via vesicular transport, of enzymes required for hydrolysis of the primary septum. It may be involved in signaling pathways that lead to the initiation of septation and to the cessation of the deposition of secondary septum material.

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bp	base pair
CAR	contractile actomyosin ring
CBD	Calcium binding domain
DAG	diacylglycerol
DHFR	dihydrofolate reductase
EDTA	ethylenedinitrilotetraacetic acid
eGFP	enhanced green fluorescence protein
EGTA	ethylene glycol tetraacetic acid
EMM	Edinburgh minimal medium
ELC	Essential light chain
ER	endoplasmic reticulum
GFP	Green fluorescence protein
IP_3	inositol 1,4,5-triphosphate
kb	kilobase pair
ME	malt extract
MLC	myosin light chain
MPF	maturation-promoting factor
MTs	microtubules
NES	nuclear export signal
NLS	nuclear localization signal
nt	nucleotide
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PH	pleckstrin homology domain
PLC	phospholipase C
Prot A	*Staphylococcus aureus* Protein A
PtdIns	phosphatidylinositol
PtdIns3P	phosphatidylinositol 3-phosphate
PtdIns4P	phosphatidylinositol 4-phosphate
PtdIns5P	phosphatidylinositol 5-phosphate
PtdIns(3,4)P_2	phosphatidylinositol 3,4-bisphosphate
PtdIns(3,5)P_2	phosphatidylinositol 3,5-bisphosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol 3,4,5-trisphosphate
SD	Yeast Synthetic Defined Medium
SDS	sodium dodecyl sulfate
SIN	Septation Initiation Network
SPA	synthetic sporulation media
SPB	spindle pole body
TAP	tandem-affinity-purification
TEM	Transmission electron microscopy
TEV	Tobacco Etch Virus
ts	temperature-sensitive

Ub	ubiquitin
YES	Yeast extract with supplements
YFP	Yellow fluorescence protein
YPD	Yeast extract peptone dextrose medium

Chapter 1: Introduction

1.1. Cytokinesis in various organisms – Overview of general and unique features

Cytokinesis is the final stage of the cell division cycle in which cytoplasm, intracellular organelles, cell membrane, cell wall and segregated chromosomes are partitioned into two daughter cells. Cytokinesis is studied in a number of eukaryotic model organisms in order to elucidate its underlying mechanisms (Nanninga, 2001; Guertin *et al.*, 2002a; Balasubramanian *et al.*, 2004). In this section, the general and unique features of cytokinesis in various organisms will be reviewed.

Generally, several steps are required for cytokinesis; determination of the cell division site, assembly and constriction of the contractile ring, cell membrane ingress at the cleavage furrow, severing of the constricted cell membrane to produce two membrane-delimited cells and hydrolysis of cell wall material to allow cell separation to occur. Astral microtubules have been implicated as the determinant for selection of the cell division site in animal cells and in the slime mold, *Dictyostelium discoideum* (Neujahr *et al.*, 1998; Robinson and Spudich, 2000). However, the position of the nucleus during early mitosis is a key factor for the selection of the cell division site in the fission yeast, *Schizosaccharomyces pombe* (Daga and Chang, 2005) as is the nucleoid in *Escherichia coli* (Margolin, 2000). Although the mechanism to decide the cell division site can differ between organisms, the underlying concept is that the division site must be selected precisely in order to prevent the production of abnormal progeny. An actomyosin ring, also known as the contractile ring, is found in the majority of eukaryotes (Hales *et al.*, 1999), except for higher plants and certain algae that utilize a transient cortical preprophase band of co-coiled bundles of microtubules and actin filaments (F-actin) (Jurgens, 2005). The basal components of the actomyosin ring are myosin II and F-actin which are found in most eukaryotes. In general, the actomyosin

ring constricts at the cell division site in coordination with new membrane insertion, and, in some organisms, with cell wall formation (Hales *et al.*, 1999). Evidence that endocytosis, exocytosis or both of these processes may be required for the insertion of new membrane or cell wall formation has been found in studies of a number of organisms including *S. pombe* (Wang *et al.*, 2002; Wang *et al.*, 2003; Gachet and Hyams, 2005), *D. discoidium* (Wienke *et al.*, 1999), *Drosophila melanogaster* (Lecuit, 2004; Strickland and Burgess, 2004; Lu and Bilder, 2005), sea urchin zygotes (*Lytechinus pictus*) (Shuster and Burgess, 2002) and *Xenopus* eggs (Danilchik *et al.*, 2003). Finally, constriction of the contractile ring and the formation of two separate compartments delimited by two independent plasma membranes, and in some cases by the cell wall, results in the formation of two daughter cells (Glotzer, 1997).

Cytokinesis has been categorized as symmetrical or asymmetrical, depending on the site of cytokinesis within the cell. Asymmetric division has been studied in multicellular organisms such as *Caenorhabditis elegans* and *Drosophila* (Betschinger and Knoblich, 2004). Asymmetric division produces two daughter cells that are not equal in size and that can have different developmental fates. The major concern of the successful asymmetrical cytokinesis is how to determine a division site and asymmetrically maintain cell fate determinants. A genetic screen for failed asymmetrical division in *C. elegans* zygotes identified PAR (*par*titioning-defective) proteins as key players. PAR proteins have a polarized distribution within the cell and are conserved among multicellular animals (Kemphues *et al.*, 1988; Ohno, 2001; Macara, 2004). Despite this, the mechanism by which asymmetry is initiated and maintained differs between organisms (Betschinger and Knoblich, 2004). For instance, to asymmetrically localize and maintain cell fate determinants, the *C. elegans* zygote utilizes PAR proteins-dependent cytoplasmic flow and ubiquitin-mediated protein degradation (Pellettieri *et al.*, 2003; DeRenzo *et al.*, 2003; Cheeks *et al.*, 2004). In contrast, *Drosophila* neuroblasts utilize protein phosphorylation (Betschinger *et al.*, 2003). This protein phosphorylation depends on PAR proteins and an atypical protein kinase C (aPKC). Despite the differences in the mechanisms in *C. elegans* and *Drosophila*, many of the key players are conserved, such as the PAR proteins, the aPKC, and the GTP-binding protein CDC42. These key players are also present in mammalian cells (Ohno,

2001; Macara, 2004). Because of this, we can anticipate that studies in these model organisms will lead to understanding of these processes in animals, including in humans.

Cytokinesis has been categorized as complete or incomplete, depending on whether daughter cells are liberated from one another or remain joined. Cytokinesis in single celled organisms must by definition be complete. Multicellular organisms often combine complete and incomplete cytokinesis.

The septum is a distinct region of the cell wall at the division site in fungi. Fungi include single celled forms, the yeasts, and multicellular forms. In yeasts, septum materials must be deposited at the site at which division will occur, and then some of the septum material must be removed by hydrolysis in order to release the daughter cells from one another. The septum of *S. pombe* is described in more detail in a subsequent section. In filamentous fungi such as *Aspergillus nidulans*, the formation of septa results in individual compartments within the hyphae. The structure of the septum in filamentous fungi is different from that of yeasts in that it includes a pore that is large enough to permit passage of some organelles. Pores can be blocked by the action of a specialized structure called 'Woronin bodies' (Walther and Wendland, 2003).

The brewing yeast, *Saccharomyces cerevisiae*, divides by budding. The budding site in *S. cerevisiae* is decided as a first step of cytokinesis before nuclear DNA synthesis starts. This is unique in that in many organisms cytokinesis takes place only after genetic materials are duplicated. A daughter bud appears when DNA synthesis is initiated and grows until the size of the daughter bud reaches approximately that of the mother cell (Pruyne *et al.*, 2004). The daughter nucleus is at last transferred to the new bud during mitosis and the daughter cell is physically separated from the mother (Guertin *et al.*, 2002a; Balasubramanian *et al.*, 2004). In particular, studies of budding suggest that the ability of the actomyosin ring to constrict may be achieved only when the septum formation is initiated, because septum formation mutants were able to assemble but unable to constrict the actomyosin ring (Bi, 2001). In budding yeast, the actomyosin ring is dispensable for cell viability but required for efficient cytokinesis. Septum formation is however essential for cell viability in budding yeast (Bi, 2001).

The mechanism for determination of the septation site in *A. nidulans* is unclear; however, it is known that actin ring formation at the future septation site

precedes septum formation (Harris, 1997). While the F-actin ring is present at the cytokinesis site, actin patches also stay at the actively growing apical tip (Harris, 1997). This contrasts to the events in yeast, in which actin distribution changes during cytokinesis. For instance, an actin dot structure (F-actin patch) is found at growing tips in fission yeast. For a period of time following cytokinesis, cell growth occurs only at the old end of the cell; that is, at the end that existed prior to the cytokinesis. Later in the cell cycle, growth continues to occur at the old end and growth initiates at the new end of the cell (Harris, 1997). During this period of bipolar growth, F-actin patches are present at both ends of the cell. At the time that the contractile ring becomes visible, actin patches are not observed at the cell ends (Figure 1.2.) (Harris, 1997; Pelham and Chang, 2002). This phenomenon also occurs in budding yeast (Harris, 1997). In *S. cerevisiae*, F-actin patches are present at the bud site and in the growing bud. However, F-actin patches are not observed during cytokinesis when the F-actin ring is observed at the bud neck site. After the F-actin ring has constricted and cytokinesis is complete, F-actin patches are visible in both mother and daughter cells. The F-actin patches in *A. nidulans* that are localized at the growing apical tip may permit cell growth to continue during cytokinesis. In all cases, a septum is formed at the site where the actin ring is assembled (Walther and Wendland, 2003). It seems that the actin ring guides septum formation at the correct site in both yeast and filamentous fungi.

Incomplete cytokinesis is also found during *Drosophila* oogenesis (Robinson *et al.*, 1994; Robinson and Cooley, 1997). Cytokinesis initiates and progresses, but it is arrested after an intracellular bridge, the ring canal, forms between germline cells at the cleavage furrow site. The ring canal is predominantly composed of actin, anillin, and other proteins, which are also found in classical cleavage furrows in which cleavage is complete (Robinson and Cooley, 1997; Guertin *et al.*, 2002a). This intracellular bridge may be important to allow transport of cytoplasm and/or cytoplasmic components for developing germline cells in *Drosophila*, because a mutant defective in ring canal assembly failed to produce a mature oocyte (Robinson *et al.*, 1994).

Cytokinesis is usually thought of as being coupled to the occurrence of the nuclear events of DNA replication and division; however, in some cases these processes can be uncoupled. In *Drosophila*, early embryogenesis is associated with uncoupled

mitosis and cytokinesis (Glotzer, 1997; Mazumdar and Mazumdar, 2002); a fertilized egg repeats rapid mitotic nuclear divisions devoid of cytokinesis for 13 cycles, producing a multinucleated single cell (syncytium). At the entry of interphase 14, cytokinesis (termed as cellularization in *Drosophila* embryogenesis) occurs in order to enclose each nucleus with plasma membrane. This indicates that cytokinesis is somehow inhibited during the first 13 mitotic cycles, and it is resumed through an unknown mechanism. A set of distinct genes is expressed during cellularization of the syncytium in *Drosophila* (Mazumdar and Mazumdar, 2002; Lecuit, 2004). Like the contractile ring components in the classical cleavage furrow, the *Drosophila* embryo furrow is based on actomyosin contractile ring components (Mazumdar and Mazumdar, 2002). The majority of the components of the contractile actomyosin ring (CAR) and the furrow are common to both structures; however, a set of proteins is specific for the furrow. For example, proteins such as *nullo*, *Serendipity-α*, *bottleneck*, and *Slow-as molasses* accumulate in late mitotic cycle 13 or early cycle 14 (Mazumdar and Mazumdar, 2002; Lecuit, 2004). *Serendipity-α* and *nullo* mutants display the disruption of actomyosin rings during cellularization and remain multinucleate, a phenotype that is characteristic of defective cytokinesis (Schweisguth *et al.*, 1990; Postner and Wieschaus, 1994).

1.2. Cytokinesis in *Schizosaccharomyces pombe*

1.2.1. The *S. pombe* life cycle – overview

The cell division cycle is a fundamental biological process. It is usually considered to have four phases, Gap1 (G1), DNA synthesis (S), Gap2 (G2), and mitosis (M, chromosome segregation). A number of cell division regulators have been identified in eukaryotes. The regulators appear to be conserved and to play similar roles in cell division control (Balasubramanian *et al.*, 2004). This is why cell cycle studies in model organisms have been able to contribute to our understanding of the cell division cycle in other organisms, including in humans.

The fission yeast, *Schizosaccharomyces pombe*, is an outstanding model for study of the eukaryotic cell cycle; its genome is completely sequenced (Wood *et al.*, 2002), it is easy and safe to handle, and well-developed and relatively simple genetic

experimental methods are available (Marks *et al.*, 1986; Moreno *et al.*, 1991). *S. pombe* is rod shaped and coated with a rigid cell wall outside of the plasma membrane. It grows by tip extension and the length changes predictably through the cell division cycle, allowing a simple estimate of the cell cycle stage that a cell may be in. *S. pombe* divides into two daughter cells by means of symmetrical medial fission. The *S. pombe* vegetative cell division cycle possesses a relatively long G2/M phase, instead of a long G1 phase as is the case in many eukaryotes. Like other fungal cell division cycles, the nuclear envelope does not break down during mitosis in *S. pombe*.

S. pombe can go through the vegetative cell division cycle as haploid cells or as diploid cells if favorable conditions are maintained such as the presence of rich nutrients. If, however, cells of opposite mating types (h^+ and h^-) are together under conditions that are favorable for sexual reproduction, such as the absence of nutrients, the gametes mate and their nuclei fuse, thus setting the stage for meiosis. After meiosis, sporulation results in the encapsulation of each haploid chromosome set, producing spores. Spores remain dormant until environmental conditions become favorable for growth. Under appropriate conditions, spores germinate and elongate producing cylindrical cells. The *S. pombe* life cycle thus alternates between vegetative mitotic and reproductive meiotic cycles, dependent on environmental conditions and the presence of cells of opposite mating types (Figure 1.1.). The following section will describe the final stage of the mitotic cycle, cytokinesis, since it is relevant to the research for this dissertation.

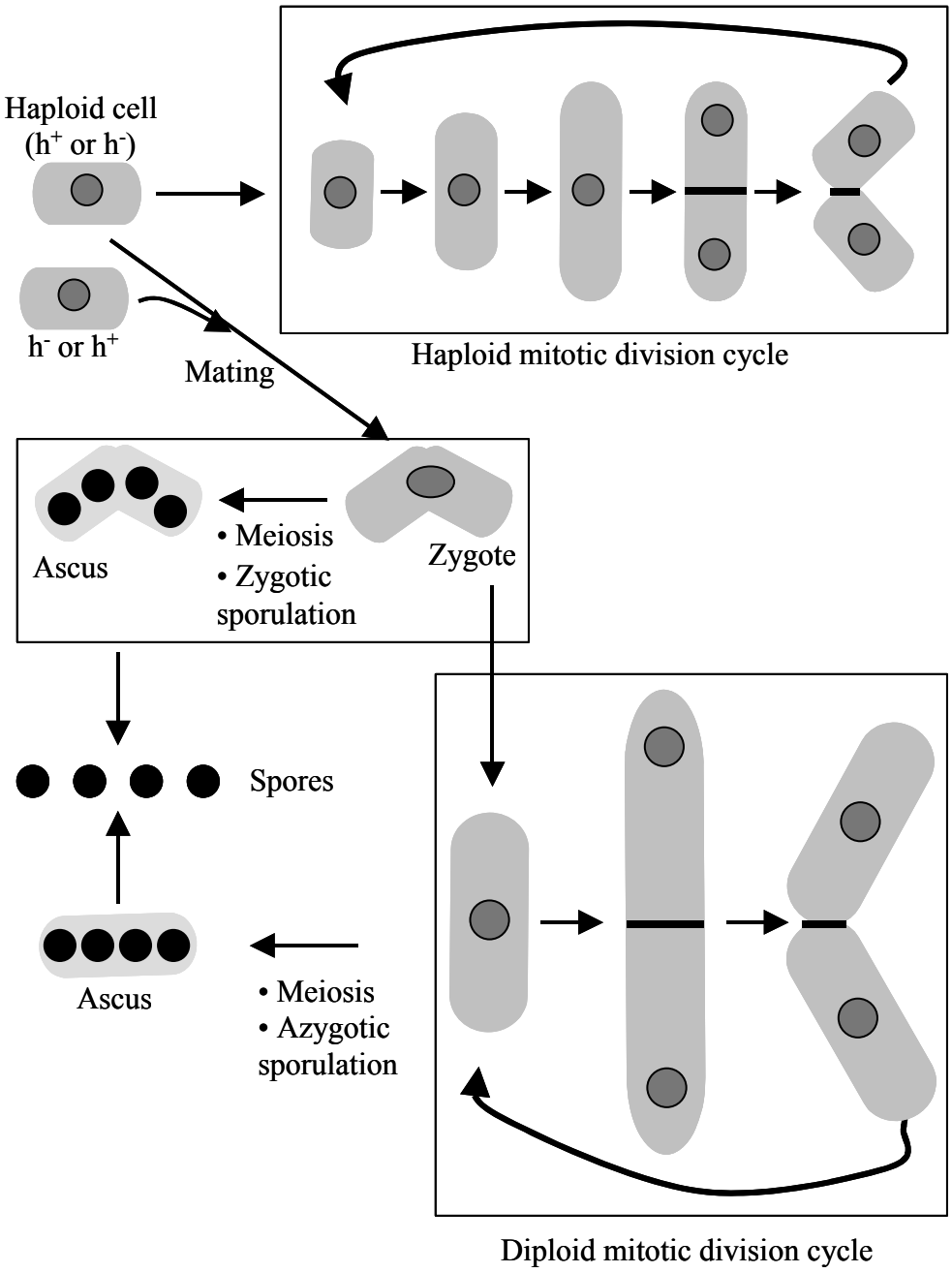


Figure 1.1. *Schizosaccharomyces pombe* life cycle. *S. pombe* goes through mitotic division cycle as haploid cells or as diploid cells. Under conditions such as a nitrogen starvation, *S. pombe* goes through the reproductive cycle of mating, meiosis, and sporulation. This is described in section 1.2.1.

1.2.2. Mechanisms of *S. pombe* cytokinesis

S. pombe cytokinesis is temporally and spatially orchestrated by a series of regulators as in other eukaryotes. Cytokinesis occurs by medial fission in coordination with actomyosin ring assembly and constriction, and septation. *S. pombe* cytokinesis is composed of the following five steps (Figure 1.2.); (1) the cell division site is first decided, (2) the components required to assemble an actomyosin ring at the division site are recruited, (3) the septum initiation pathway is activated, (4) new plasma membrane is inserted and septum layers (primary and secondary) are formed at the division site, (5) once new plasma membrane and septum are successfully formed between completely segregated nuclei, the primary septum is hydrolysed and the daughter cells are liberated.

The identification and study of *S. pombe* genes involved in cytokinesis was initiated with the first isolation of *S. pombe* cell division control mutants by Nurse and colleagues over 30 years ago (Nurse *et al.*, 1976; Chang *et al.*, 1996; Balasubramanian *et al.*, 1998). Table 1.1. lists the *S. pombe* cytokinesis proteins and their mutant phenotypes.

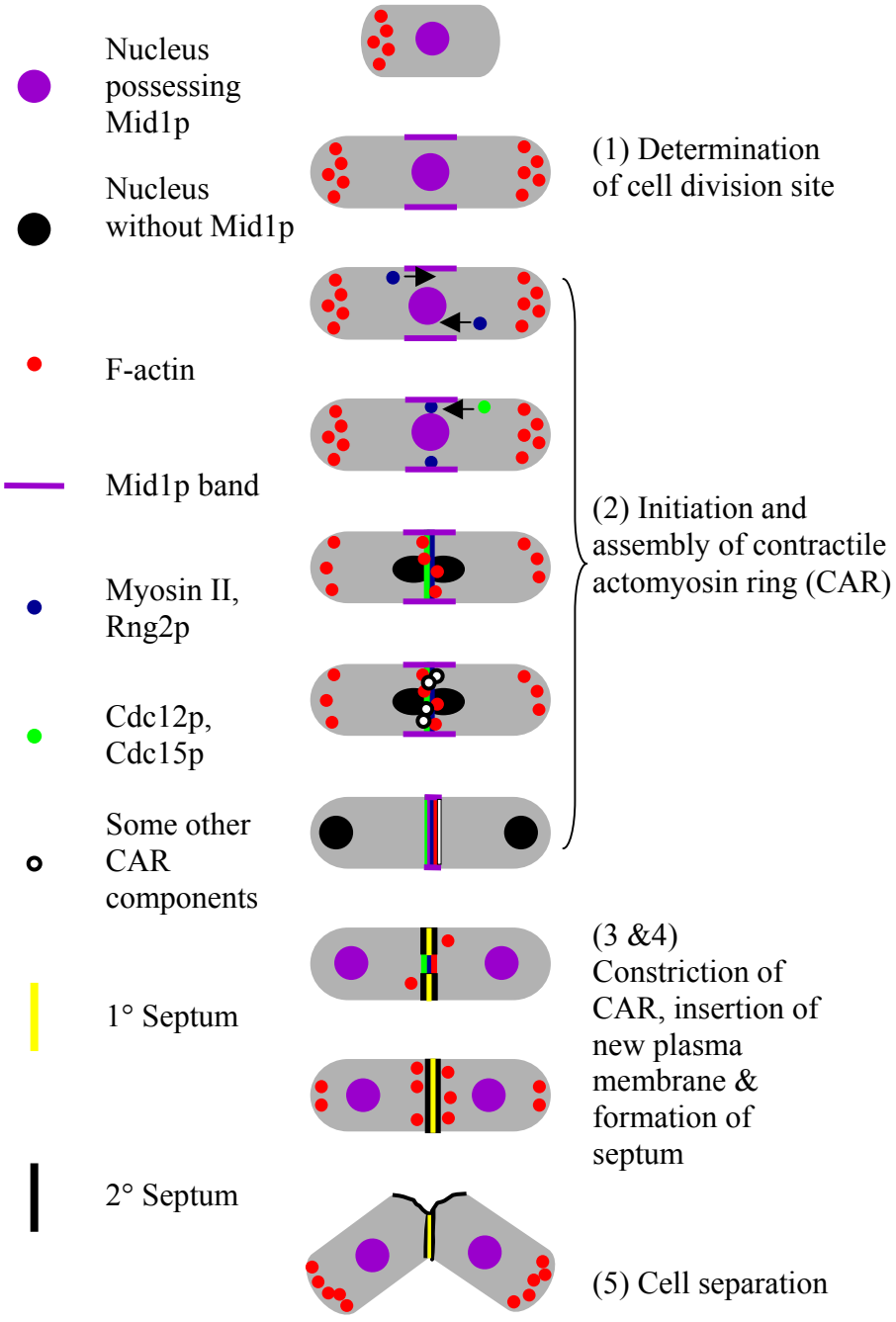


Figure 1.2. *Schizosaccharomyces pombe* cytokinesis. Schematic cytokinesis steps are drawn: determination of cell division site, initiation and assembly of contractile actomyosin ring, constriction of contractile ring and formation of septum, and cell separation. These steps are described in detail in section 1.2.2.

Table 1.1. Cytokinesis components in *S. pombe*

		Cell viability	Loss-of-function	Reference
Determination of cell division site				
Mid1p	anillin-like protein	non-essential	Division plane displacement	Sohrmann *et al.*, 1996
Plo1p	Polo-like protein	essential	Division plane displacement	Bahler *et al.*, 1998
Initiation/assembly of actomyosin contractile ring formation				
Myo2p	Myosin II heavy chain	essential	Cell elongation with multinuclei, No/defective CAR formation, aberrant septum formation	Balasubramanian *et al*., 1998
Cdc4p	Myosin II essential light chain	essential	Cell elongation with multinuclei, No/defective CAR formation, aberrant septum formation	McCollum *et al.*, 1995
Rlc1p	Myosin II regulatory light chain	non-essential	Cell elongation with multinuclei, No/defective CAR formation, aberrant septum formation	Naqvi *et al.*, 2000
Rng2p	IQGAP	essential	F-actin cable organization defect	Eng *et al*., 1998
Rng3p	UCS protein	essential	Cell elongation with multinuclei, No/defective CAR formation, aberrant septum formation	Lord and Pollard, 2004
Cdc15p	PCH protein	essential	Cell elongation with multinuclei, No CAR/septum formation, Desruption of a sterol-rich membrance domain organization	Takeda *et al*., 2004
Cdc3p	profilin	essential	No CAR formation, aberrant septal material deposition, disorganization of F-actin patches during cell cycle	Balasubramanian *et al*., 1994
Cdc12p	formin	essential	No CAR formation, aberrant septal material deposition, abnormal F-actin patch distribution in mitosis, Multinucleate	Chang *et al*., 1997
Cdc8p	tropomyosin	essential	Cell elongation with multinuclei, No/defective CAR formation, aberrant septum formation	Balasubramanian *et al*., 1992
Myp2p	Myosin II heavy chain	non-essential	No CAR contraction	Mulvihill and Hyams, 2003

Table 1.1. Cytokinesis components in *S. pombe* (continued)

		Cell viability	Loss-of-function	Reference
Septation initiation network (SIN)				
Cdc11p	Coiled-coil scaffold	essential	Multinuclei, No septum formation	Krapp *et al*., 2001
Sid4p	Coiled-coil scaffold	essential	Multinuclei, No septum formation	Balasubramanian *et al*., 1998
Spg1p	GTPase protein	essential	Multinuclei, No septum formation	Sohrmann *et al*., 1998
Cdc7p	protein kinase	essential	Multinuclei, No septum formation	Sohrmann *et al*., 1998
Cdc16p	GAP	essential	Multiple septa formation	Furge *et al*., 1999
Byr4p	GAP scaffold	essential	Multiple septa formation	Furge *et al*., 1999
Sid1p	protein kinase	essential	No septum formation	Guertin *et al*., 2000
Cdc14p		essential	Multinuclei, No septum formation	Guertin *et al*., 2000
Sid2p	protein kinase	essential	No septum formation	Hou *et al*., 2004
Mob1p		essential	Septum formation defect	Hou *et al*., 2004
Dma1p	FHA-RING finger protein	non-essential	Aberrant septation	Murone and Simanis, 1996
Clp1p	CDC14-like phosphatase	non-essential	Semi-wee	Trautmann *et al*., 2001

Table 1.1. Cytokinesis components in *S. pombe* (continued)

		Cell viability	Loss-of-function	Reference
Septation & Cell separation				
Cps1p	β-1,3-glucan synthase catalytic subnuit	essential	Cells arrested with binuclei, No septum formation	Liu *et al*., 2002
Agn1p	endo-α-1,3-glucanase	non-essential	Cell separation defect	Dekker *et al*., 2004
Eng1p	endo-β-1,3-glucanase	non-essential	Cell separation defect	Dekker *et al*., 2004
Sec8p, Sec6p, Sec10p, Sec15p, Exo70p	exocyst	essential, except Exo70p	Cell separation defect	Wang *et al*., 2002
Rho3p	Rho family GTPase	non-essential	Cell separation defect	Wang *et al*., 2003
Rho4p	Rho family GTPase	non-essential	Cell separation defect	Santos *et al*., 2005
Mid2p	anillin-like protein	non-essential	Cell separation defect	Berlin *et al*., 2003
Spn1p, Spn2p, Spn3p, Spn4p	septins	non-essential	Cell separation defect	Berlin *et al*., 2003
Myo52p	Myosin V heavy chain	non-essential	Septation failure	Mulvihill *et al*., 2006

1.2.2.1. Determination of cell division site

Fission occurs in the middle of the long axis of the *S. pombe* cell. Important questions include: when and how is the site for fission determined during the cell division cycle? It was found that the position of the interphase nucleus is critical for the determination of the cell division site by experimental displacement of the interphase nucleus by centrifugation (Daga and Chang, 2005; Burgess and Chang, 2005). The position at which the contractile ring was formed was monitored by observing the distribution of a fluorescently tagged component of the ring; the myosin II regulatory light chain (Rlc1p-GFP) using time-lapse microscopy (Daga and Chang, 2005). *S. pombe* Rlc1p is localized at the division site as a component of the actomyosin contractile ring (CAR) during cytokinesis (Naqvi *et al.*, 2000; Wu *et al.*, 2003) (See section 1.2.2.2.). The Rlc1p-GFP was displaced to the end of the cell where the interphase nucleus resided (Daga and Chang, 2005). On the other hand, its localization was not affected by centrifugation when the nucleus was displaced from the center in mid-mitosis: the Rlc1p-GFP was detected in the middle of most cells (Daga and Chang, 2005). Unlike animal cells in which microtubules (MTs), especially astral MTs, affect the determination of their division sites (Guertin *et al.*, 2002a; Balasubramanian *et al.*, 2004; Burgess and Chang, 2005), the MTs in *S. pombe* do not seem to directly involve the determination of the division site. The MTs rather appear to position the nucleus at the center of *S. pombe* cell (Tran *et al.*, 2001). Treatments that produced MT depolymerization resulted in failure of the nucleus to relocate to the center of the cell, finally resulting in the displacement of the division site to wherever the nucleus resided (Tran *et al.*, 2001). Furthermore, *S. pombe* astral MTs appear after the CAR is established (Chang *et al.*, 1996; Burgess and Chang, 2005).

S. pombe mid1 was identified as a key landmark for the division site but it is nonessential for cell viability (Sohrmann *et al.*, 1996; Chang *et al.*, 1996). *mid1* encodes an anillin-like protein. Anillin has been reported to act as a landmark for the site of the cleavage furrow in dividing human cells and in *Drosophila* cells during cellularization (Giansanti *et al.*, 1999; Oegema *et al.*, 2000). *mid1* mutants were defective in the placement and assembly of the CAR and septum (Sohrmann *et al.*, 1996; Chang *et al.*, 1996; Paoletti and Chang, 2000). The *S. pombe mid1* gene encodes a phosphoprotein

that possesses a nuclear-localization signal (NLS), two nuclear export signals (NES), and a carboxy-terminal pleckstrin homology (PH) domain (Sohrmann *et al.*, 1996; Paoletti and Chang, 2000). NLS and NES are known to be involved in nuclear shuttling (Moroianu, 1999). PH domains bind phosphoinositides. Phosphoinositides are phosphorylated forms of the membrane phospholipid, phosphatidylinositol, and some phosphoinositides are key signaling molecules (Overduin *et al.*, 2001). Mid1p localization changes during the cell cycle (Figure 1.2.) (Sohrmann *et al.*, 1996; Paoletti and Chang, 2000). In cells in mid to late G2, Mid1p is found in the nucleus and in a broad, medial cortical area surrounding the nucleus. In early mitotic cells, the nuclear localization is lost and Mid1p appears only as the broad, medial cortical band. In anaphase cells, the broad Mid1p band becomes compacted into a tight medial ring. In septating and G1/S cells, Mid1p is found only in the nucleus. Mid1p is hyperphosphorylated at the time that it is translocated to the broad, medial cortical area coincident with the onset of mitosis, when it is considered to be active (Sohrmann *et al.*, 1996). This suggests that Mid1p function may be regulated by its cellular distribution as well as by post-translational modification.

The presence of NLS and NES sequences in Mid1p indicates that it might shuttle in and out of the nucleus, and the presence of the PH domain suggests that Mid1p may associate with the medial cortical membrane. To investigate if Mid1p domains are essential for Mid1p localization during the cell cycle and for its functions, Paoletti and Chang created strains carrying a *mid1* deletion allele and containing a second *mid1* allele with an NES deletion, or an NLS mutation, or a PH domain deletion (Paoletti and Chang, 2000). The NES deletion or NLS mutation disrupted nuclear shuttling; however, these alleles restored the division site selection and septum formation defects of the *mid1*-null mutant. The evidence for this restoration was the observation of a faint medial cortical band of Mid1 detected by indirect immunofluorescence microscopy. The medial septum formed, with some abnormalities. The PH domain was nonessential for Mid1p function and for its translocation to the cell cortex. Mid1p lacking the PH domain was localized in the same manner as wild-type Mid1p. On the basis of these studies, it was suggested that the PH domain function is not required for the stabilization of Mid1p anchoring to the cortical membrane and the NES and NLS are not required for efficient nuclear

shuttling (Celton-Morizur *et al.*, 2004). On the other hand, the C-terminal PH domain of human anillin is required for the localization to the cortical cleavage furrow (Giansanti *et al.*, 1999; Oegema *et al.*, 2000). It is still unclear how and what assists Mid1p to shuttle between the nucleus and the cytoplasm during cell division cycle, and what regulates its activity (phosphorylation/dephosphorylation).

Plo1p is a Polo-like Ser/Thr kinase, that has been proposed as a regulator for Mid1p localization and function in *S. pombe* (Bahler *et al.*, 1998; Paoletti and Chang, 2000; Mulvihill and Hyams, 2002; Anderson *et al.*, 2002). Polo was first isolated in a screen of *Drosophila* mutants causing abnormal mitosis (Llamazares *et al*., 1991). It is conserved in yeast and humans (Polo-like kinases, Plks) (Lee *et al*., 2005). Studies of Polo-like kinases in many organisms indicate that they are involved in multiple stages of the cell cycle; such as, the G2/M and metaphase/anaphase transitions and cytokinesis (Lee *et al*., 2005). *S. pombe plo1* was originally isolated in a genetic screen for other mutants displaying the *mid1* defective phenotype (Bahler *et al.*, 1998). Cdc4p is a myosin II essential light chain and an essential CAR component (McCollum *et al.*, 1995; Desautels *et al.*, 2001). The location of GFP-tagged Cdc4p and of septum material was observed in cells carrying *mid1-18* or *plo1-1*, temperature-sensitive (ts) alleles (Bahler *et al.*, 1998). The Cdc4p-GFP ring and septum were displaced in both mutants at the restrictive temperature. Further studies indicated physical and genetic interactions between *mid1* and *plo1*. A yeast two-hybrid assay showed that Plo1p interacts with Mid1p through the C-terminal half of Plo1p (amino acids 320 – 683) (Bahler *et al.*, 1998). In *plo1-1* cells at the restrictive temperature, Mid1p was found during anaphase to be predominantly localized to the nucleus (Bahler *et al.*, 1998), with some Mid1p also found as the broad medial band (Paoletti and Chang, 2000). However, the tight band of Mid1p was not observed in *plo1-1* cells under these conditions (Paoletti and Chang, 2000). Mid1p was not observed to be associated with nuclei when *plo1* was overexpressed. Overexpression of *plo1* seemed to maintain Mid1p in a phosphorylated state (Bahler *et al.*, 1998). The Plo1p cellular localization was also affected in a *mid1*-deletion mutant. In wild-type cells, Plo1p is found on spindle pole bodies (SPBs), mitotic spindles, and at the medial division site. In the *mid1*-deletion mutant, Plo1p was detected on mitotic SPBs and mitotic spindles but not at the medial division site (Bahler

et al., 1998). These results suggest that Plo1p may directly or indirectly regulate the transport of nuclear Mid1p to the cortical broad band and the conversion of the broad band of Mid1p to the tight band. Plo1p may also regulate Mid1p activity by phosphorylation. The function of Mid1p is required for the localization of Plo1p to the medial division site.

1.2.2.2. Initiation and assembly of contractile actomyosin ring

Once the division site is selected by the interphase nucleus and Mid1p, the CAR components are recruited and assembled at the division site (Table 1.1.) (Balasubramanian *et al.*, 2004). To understand this process more fully, several questions have to be addressed; [1] what is the order of CAR component recruitment? [2] how are CAR components organized at the medial plane? and [3] how is the initial assembly of the recruited CAR components achieved?

Wu *et al*. addressed the first question, the temporal sequence of CAR component recruitment, by time-lapse microscopy of cells in which various CAR proteins were fused to GFP, YFP or CFP (Wu *et al.*, 2003). Before the onset of mitosis, the myosin II heavy chain, Myo2p; the essential light chain, Cdc4p; the regulatory light chain, Rlc1p; and IQGAP Rng2p are recruited to the medial broad band defined by the presence of Mid1p. At the onset of mitosis, Cdc15p and Cdc12p are recruited to the same region. The medial localization of these 6 CAR proteins and of Mid1p is achieved in an actin-independent manner since treatment with the actin depolymerizing drug, Latrunculin A (Lat-A), did not affect their localization to the medial broad band (Wu *et al.*, 2003). A schematic illustration of these events is presented in Figure 1.2. The phenotypes induced by loss-of-function of each of the CAR proteins are listed in Table 1.1. Despite the determination of the temporal order of recruitment of the CAR proteins, how they are initially assembled to become a tight contractile ring remains controversial. One hypothesis is that a cable filament, which contains Myo2p, Cdc4p, Rlc1p, Cdc12p, and Cdc15p, grows from a medial progenitor spot that contains those proteins (Wong *et al.*, 2002; Hou and McCollum, 2002; Carnahan and Gould, 2003). Another hypothesis is that the puncta containing Mid1p, Myo2p, Cdc4p, Rlc1p, Cdc12p, Cdc15p and Rng2p are accumulated at the medial broad band and then laterally compacted into a fine

contractile ring (Wu *et al.*, 2003; Wu *et al.*, 2006). Whatever is the initial assembly mechanism, the transition to a tight compact ring depends on the actin cytoskeleton (Mulvihill and Hyams, 2002; Wu *et al.*, 2003). The maintenance of the myosin II ring after its assembly does not require the actin cytoskeleton (Naqvi *et al.*, 1999). The association of some CAR proteins with the ring is dependent on polymerized actin (Wu *et al.*, 2003). These are the F-actin binding proteins: tropomyosin, Cdc8p; α-actinin, Ain1p; capping protein, Acp2p; and unconventional Myosin-II heavy chain, Myp2p. Thus, the initial recruitment and assembly of the CAR components are temporally regulated in coordination with the actin cytoskeleton.

The spatial organization of the CAR components during recruitment to the medial region and during assembly of the CAR is still unclear. Several individual studies however indicated the following: Mid1p interacts with Myo2p (Motegi *et al.*, 2004); Myo2p interacts with Cdc4p and Rlc1p (Le Goff *et al.*, 2000); Cdc4p interacts with Rng2p, Myo2p, and Myp2p (Le Goff *et al.*, 2000; D'souza *et al.*, 2001); and Cdc12p interacts with Cdc15p and Cdc3p (Chang *et al.*, 1997; Carnahan and Gould, 2003). The significance of the Cdc12p, Cdc15p, and Cdc3p recruitment to the medial broad band is the stimulation of actin polymerization (Wu *et al.*, 2003; Wu *et al.*, 2006).

Despite the studies of the temporal and spatial regulation of the actomyosin ring components in *S. pombe*, it is still unclear how the actomyosin ring is associated with the plasma membrane.

1.2.2.3. Septation initiation network (SIN)

The medial Mid1p disassociates from the medial cortex when the actomyosin ring starts to constrict (Wu *et al*., 2003). The constriction of the CAR and the accumulation of septum materials are regulated by a signal transduction pathway termed the Septation Initiation Network (SIN) (Simanis, 2003; Krapp *et al.*, 2004b). The SIN pathway is comprised of a number of proteins (Table 1.1.), including three protein kinases (Cdc7p, Sid1p, and Sid2p) and one small GTPase (Spg1p). SIN mutants can form the actomyosin ring, but fail to constrict the ring, resulting in mutilucleated cells (Balasubramanian *et al.*, 1998). The actomyosin rings in the SIN mutants tend to fall apart after they are assembled. In contrast, in wild-type cells, after the ring is assembled

it persists for a period with a constant diameter while some late events occur, such as the recruitment of Myp2p (Wu *et al.*, 2003). This indicates that the SIN pathway is required both for maintenance of the actomyosin ring in late anaphase and for its constriction. Aberrant septum material accumulation was observed in cells containing an overexpressed SIN regulator (Sohrmann *et al.*, 1998). Thus, in addition to its roles in maintenance and contraction of the CAR, the SIN pathway appears to be involved in the regulation of septum formation

The *S. pombe* SPB is a microtubule-organizing center (MTOC) that is homologous to the mammalian centrosome. Duplicated SPBs separate at the onset of mitosis (Wu *et al.*, 2003), leading chromosome segregation. In fission yeast, the role of the SPB is not only that of an MTOC but it is also involved in the regulation of the SIN pathway. The SIN signaling pathway is first commenced by SIN components on the SPB (McCollum and Gould, 2001; Bardin and Amon, 2001; Simanis, 2003; Krapp *et al.*, 2004b). During cytokinesis, some SIN components are present on both SPBs while some components are restricted to only one of the two SPBs (Figure 1.3. A) (Simanis, 2003). Cdc11p, an essential gene for cell viability, physically interacts with Sid4p to play a role as a platform for the loading of other SIN components (Morrell *et al.*, 2004). The Cdc11p-Sid4p complex symmetrically resides on SPBs during cytokinesis (Morrell *et al.*, 2004). Figure 1.3. B demonstrates the SIN pathway. Cdc11p is highly phosphorylated during anaphase when the SIN pathway is active (Krapp *et al.*, 2001). Cdc11p interacts with Cdc13p, which is the cyclin B component of maturation-promoting factor (MPF). Therefore, phosphorylation of Cdc11p by MPF was investigated (Morrell *et al.*, 2004). The eight potential MPF-phosphorylation consensus residues (serines) near the amino terminus of Cdc11p were mutated to alanine to mimic constant dephosphorylation (Morrell *et al.*, 2004). This *cdc11* mutant was still functional as it complemented a *cdc11*-null allele (Morrell *et al.*, 2004). Cdc11p phosphorylation instead seems to require the activity of the protein kinase, Cdc7p, even though it is unclear whether Cdc7p directly phosphorylates Cdc11p (Krapp *et al.*, 2003). Cdc7p interacts with GTP-Spg1p, which also associates with the N-terminal region of Cdc11p (Sohrmann *et al.*, 1998). The GTP-Spg1p-Cdc7p complex appears in early mitosis (Guertin *et al.*, 2000; Simanis, 2003). The GTPase activity of Spg1p is regulated by the Byr4p-Cdc16p

complex, which is a GTPase-activating protein (GAP) (Fournier *et al.*, 2001) (Figure 1.3. B). Byr4p can simultaneously interact directly with both Spg1p and Cdc16p (Jwa and Song, 1998; Furge *et al.*, 1999). Byr4p constitutively resides in SPBs during the cell cycle, whereas Cdc16p appears in SPBs in early anaphase where it remains until the onset of the next mitosis (Cerutti and Simanis, 1999). *In vitro* in the absence of Cdc16p, Spg1p is inactive as a GTPase, and Byr4p dissociates from it (Furge *et al.*, 1998). This suggests that Byr4p may have a dual role, first to activate Spg1p GTPase activity (GTP-Spg1p to GDP-Spg1p) by acting together with Cdc16p, and secondly to inactivate Spg1p GTPase activity when acting alone. GTP-Spg1p triggers the SIN signal transduction pathway (Schmidt *et al.*, 1997). As demonstrated above, GTP-Spg1p on one of the two SPBs is altered to GDP-Spg1p by the activity of the Byr4p-Cdc16p complex and Cdc7p is consequently disassociated from GDP-Spg1p. *In vitro* only the GTP-bound form of Spg1p interacts with Cdc7p (Sohrmann *et al.*, 1998). The distribution of the GTP-Spg1p-Cdc7p and Byr4p-Cdc16p complexes on SPBs thus becomes asymmetrical (Figure 1.3. A) (Sohrmann *et al.*, 1998). The question why the complexes have to be asymmetrically distributed on SPBs remains uncertain. The overexpression of Spg1p induces septum formation at any stage of the cell cycle (Schmidt *et al.*, 1997; Guertin *et al.*, 2002b), suggesting that Spg1p is sufficient for triggering septation. Cdc7p acts downstream of Spg1p (Schmidt *et al.*, 1997) since cells with an increased level of Cdc7p no longer require Spg1p for activation of the SIN pathway. The Sid1p-Cdc14p complex is found on one of the two SPBs, the one on which the GTP-Spg1p-Cdc7p complex resides (Guertin *et al.*, 2000). In addition to the presence of the GTP-Spg1p-Cdc7p complex on the SPB, the recruitment of the Sid1p-Cdc14p complex to the SPB requires inactivation of MPF (Cdc2p kinase inactivation and Cdc13p degradation) (Guertin *et al.*, 2000; Simanis, 2003) (Figure 1.3. B). The Sid1p-Cdc14p complex appeared on the SPB in *cdc2* or *cdc13* ts mutant cells in a restrictive temperature, whereas it was not detected on the SPB at the permissive temperature when MPF was active. This suggests that the SIN pathway is initiated from the SPBs and coupled with inactivation of MPF. Interestingly, none of the SIN proteins mentioned above (Cdc11p, Sid4p, Spg1p, Cdc7p, Byr4p-Cdc16p, and Sid1p-Cdc14p) appears at the medial division plane (Simanis, 2003). The remaining question is how the SIN signaling pathway finally transduces a cue signal

to the actual division plane for the actomyosin ring constriction and septum material deposition. The possible final executor for these processes is the Sid2p kinase (Figure 1.3. B). The Sid2p kinase is found on the SPBs at all stages of cell cycle (Sparks *et al.*, 1999). It is however transiently localized to the division site in late anaphase. The medial Sid2p ring does not constrict (Sparks *et al.*, 1999). Its localization as well as the regulation of its kinase activity requires the interaction with Mob1p (Hou *et al.*, 2004). Truncated forms of Sid2p were unable to interact with Mob1p and failed to localize to the SPBs or to the medial division site. The kinase activity of Sid2p and its interaction with Mob1p were diminished in *mob*1 ts mutant cells at the restrictive temperature. The activities of other SIN proteins are also required for the localization of Sid2p to the SPBs and to the division site as determined by observation of GFP-tagged Sid2p in cells carrying various SIN mutants (Sparks *et al.*, 1999). The localization of Sid2p to the division site also depends on the actomyosin ring, since Sid2p failed to localize to the division site in cells carrying alleles of *cdc3* and *cdc15* that block the assembly of the actomyosin ring (Sparks *et al.*, 1999). The deposition of septum material induced by overexpression of *spg1* requires the activity of *sid2* since it did not occur in *sid2* mutant cells (Sparks *et al.*, 1999). Sid2p appears to be the last effecter of the SIN pathway, and its localization and function depend on protein-protein interaction, other SIN proteins and the actomyosin ring, indicating that *S. pombe* Sid2p may temporally and spatially determine the ring constriction and septum material deposition. However, the substrate for Sid2p kinase activity remains to be determined.

The SIN pathway is not only regulated by the SIN proteins, but also by other regulators such as Plo1p, Dma1p, and Clp1p (Simanis, 2003) (Figure 1.3. B). The *S. cerevisiae* Spg1p homologue, Tem1p, is also negatively regulated by an *S. cerevisiae* GAP, the Bfa1p-Bub2p complex, which is homologous to the Byr4p-Cdc16p complex (Simanis, 2003). The GAP activity of the Bfa1p-Bub2p complex is inactivated by the *S. cerevisiae* Polo-like kinase, Cdc5p (Hu *et al.*, 2001). Cdc5p inactivates Bfa1p by phosphorylation which allows cells to exit from mitosis (Hu *et al.*, 2001). *S. pombe*, Plo1p is also known to be involved in the SIN pathway, since *plo1* overexpression, like *spg1* or *cdc7* overexpression, induces septum formation at any stage of the cell cycle (Ohkura *et al.*, 1995; Sohrmann *et al.*, 1998). Plo1p overproduction causes the

recruitment of Cdc7p to SPBs and eventually induces septation even in cells in G2 (Mulvihill *et al.*, 1999). However, whether Plo1p phosphorylates and inactivates Byr4p (the *S. cerevisiae* Bfa1p homologue) remains to be determined. However, it must not be discounted that Plo1p may also regulate Cdc16p. When Plo1p activity was very strong in early mitosis and its signal was highly condensed at the SPBs, Cdc16p activity and signal were not detectable at the SPBs (Mulvihill *et al.*, 1999; Cerutti and Simanis, 1999; Simanis, 2003). This suggests that the Plo1p function(s) may somehow antagonize Cdc16p function(s) in early mitosis. Plo1p interacts with Sid4p (Sid4p has no apparent *S. cerevisiae* orthologue) (Morrell *et al.*, 2004) and its activity is also required for the phosphorylation of Cdc11p (Krapp *et al.*, 2004a). These results indicate that Plo1p may be an early positive SIN regulator.

S. pombe dma1 negatively regulates the SIN pathway (Murone and Simanis, 1996; Guertin *et al.*, 2002b) (Figure 1.3. B). Dma1p localizes to the SPBs, the division plane, or to both locations in a cell cycle-dependent manner (Guertin *et al.*, 2002b). It associates with the SIN scaffold protein, Sid4p, (Guertin *et al.*, 2002b). A *dma1* null allele shows aberrant septation without anaphase completion, whereas overexpression of *dma1* induces cell elongation and results in cells with multiple nuclei (Murone and Simanis, 1996). Overexpression of *dma1* affects the localization of some SIN components to the SPBs; Cdc7p, Sid1p, and Sid2p were delocalized from the SPBs, but Sid4p, Cdc11p, and Spg1p were not (Guertin *et al.*, 2002b). Overexpression of *dma1* also blocks the localization of Plo1p onto the mitotic SPBs in wild type cells (Guertin *et al.*, 2002b). Dma1p appears to be a ubiquitin-protein ligase E3 (Murone and Simanis, 1996). Thus, the disappearance of Plo1p from the SPBs upon overexpression of *dma1* might be due to proteolysis (Guertin *et al.*, 2002b). Overexpression of *dma1* did not affect the level of Plo1p during the cell cycle (Guertin *et al.*, 2002b), thus the delocalization of Plo1p from the SPBs may not be due to proteolysis of Plo1p. Rather, Dma1p may directly or indirectly affect the interaction of Plo1p with other proteins at the SPB. In either case, Dma1p seems to inhibit SIN activity by regulating Plo1p localization to the SPBs. However, Dma1p does not seem to be the sole negative regulator of the SIN pathway. On the basis of the observation of the localization of fluorescently tagged Plo1p in cells overexpressing *spg1* or *cdc7*, or cells carrying loss-

of-function alleles of *spg1* or *cdc7*, neither the initiation nor the inactivation of septation requires the association of Plo1p with the SPBs (Mulvihill *et al.*, 1999).

S. pombe clp1 is non-essential for cell viability (Trautmann *et al.*, 2001; Cueille *et al.*, 2001) and it appears to be conserved through evolution (Cueille *et al.*, 2001; Stegmeier and Amon, 2004; Vazquez-Novelle *et al.*, 2005). The localization and roles of its orthologue in *S. cerevisiae*, Cdc14p, have been studied (Stegmeier and Amon, 2004). Cdc14p is a protein phosphatase whose distribution varies in a cell cycle-dependent manner. It is found at different phases in the nucleolus, the nucleus, the SPBs and in the cytoplasm. It is involved in regulating exit from mitosis through the inactivation of the mitotic cyclin-dependent kinase complex. In *S. pombe*, the subcellular localization of Clp1p is also dynamic (Trautmann *et al.*, 2001; Cueille *et al.*, 2001; Simanis, 2003). In interphase, Clp1p was found in the nucleolus and the SPBs. As mitosis was initiated, Clp1p was dispersed in the nucleus, and was still present in the nucleolus as well as on the SPBs. During cytokinesis, Clp1p was localized at the mitotic spindle, the division site, and the SPBs. The medial Clp1p was associated with the CAR. It constricted when the CAR contracted. The dynamic localization of Clp1p suggests that it may be involved in numerous processes during cell division. A *clp1*-null mutant exhibited strong genetic interactions with the SIN mutants. For instance, the overwhelming septum formation induced by overexpression of *spg1* or by loss of function of *cdc16* was partially suppressed in cells carrying a *clp1* deletion allele. These data suggest that *S. pombe clp1* plays a role in the SIN pathway (Figure 1.3. B). How *S. pombe clp1* regulates the SIN pathway still needs to be investigated. One possibility suggested is that Clp1p indirectly inhibits Cdc2p activity through regulating the phosphorylation status of the Cdc2p activator, Cdc25p, or the Cdc2p inhibitor, Wee1p (Trautmann *et al.*, 2001; Esteban *et al.*, 2004). Sid1p localization was dependent on the presence of Clp1p (Trautmann *et al.*, 2001). Sid1p is known to localize onto SPBs when MPF is inactivated, but Cdc7p is not affected by MPF activity (Guertin *et al.*, 2000). It may be possible that Clp1p keeps the Cdc2p activity low in order for Sid1p to localize onto the SPBs, resulting in the execution of septation initiation. However, what releases *S. pombe* Clp1p from the nucleolus at the time of mitosis, and the identity of the Clp1p substrate at the division site and SPBs during cytokinesis are still uncertain.

A

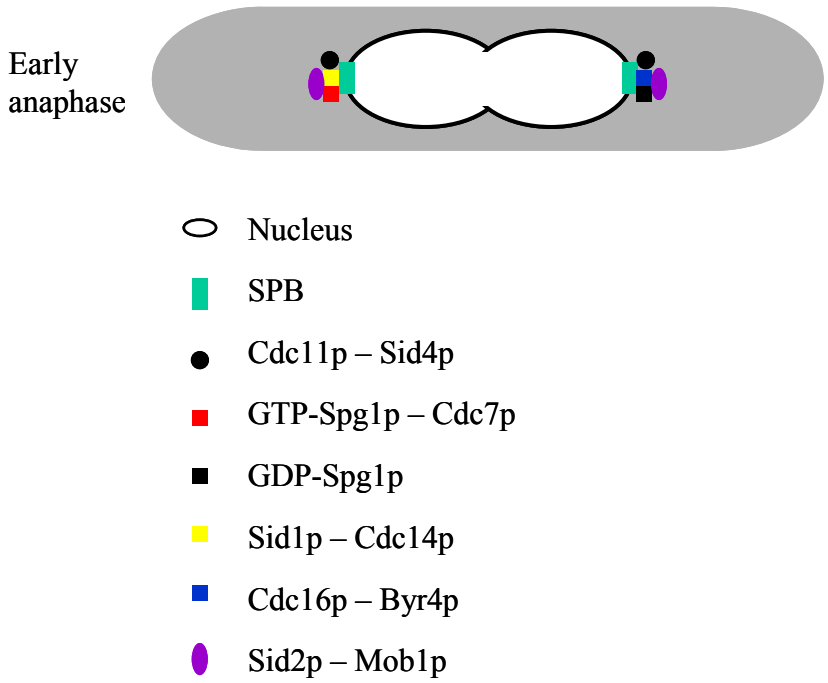


Figure 1.3. Septation Initiation Network (SIN). (A) Schematic localizations of SIN components in early anaphase. Some components such as GTP-Spg1p-Cdc7p, GDP-Spg1p, Sid1p-Cdc14p, and Cdc16p-Byr4p are asymmetrically localized in SPBs, but not Cdc11p-Sid4p and Sid2p-Mob1p. (B) Schematic SIN pathway. This is fully described in section 1.2.2.3. Red designates unidentified pathways. Black designates known pathways.

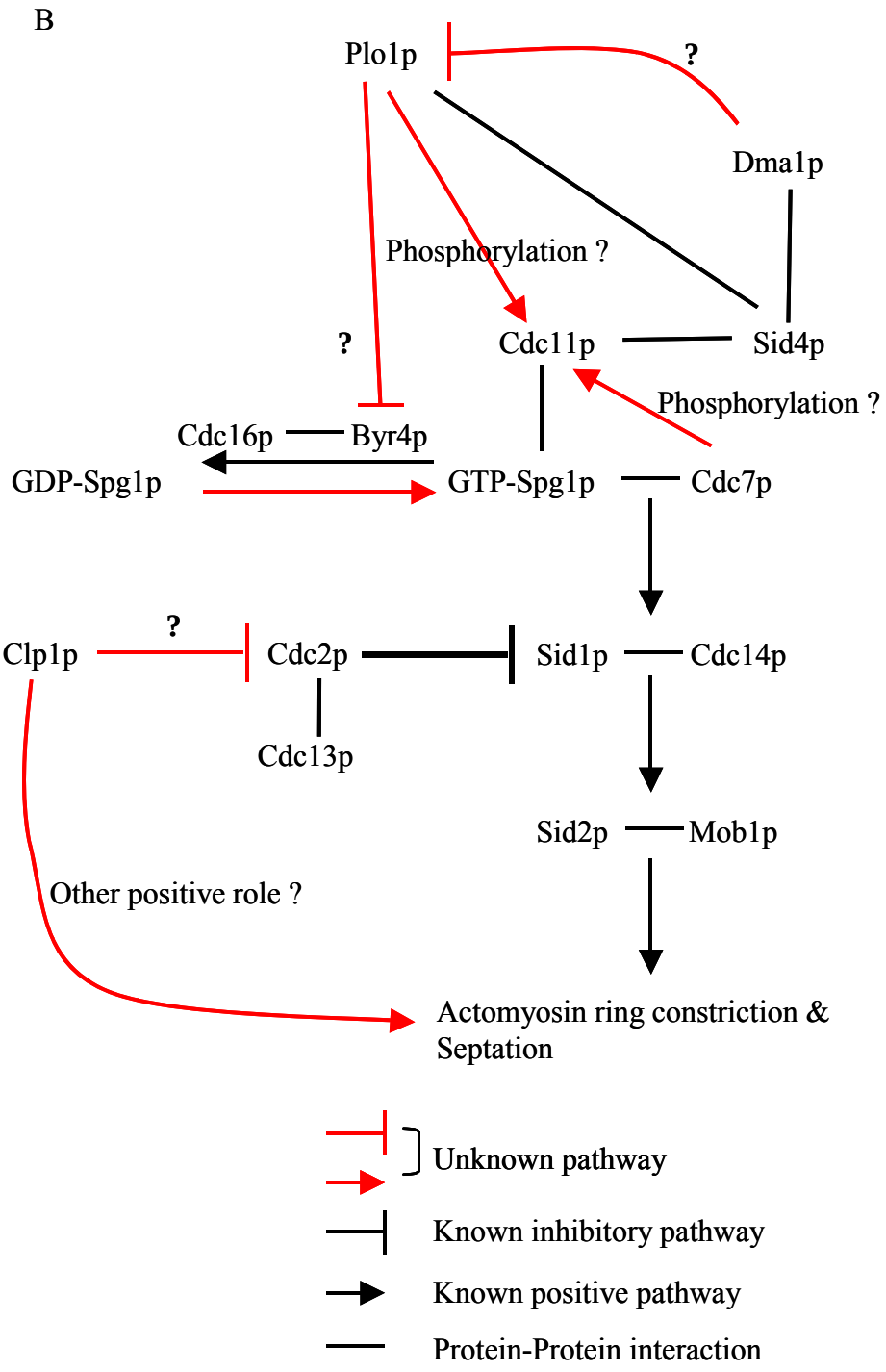


Figure 1.3. Septation Initiation Network (SIN) (continued)

1.2.2.4. Membrane expansion and septum formation

As in other eukaryotes, *S. pombe* cytokinesis is also accompanied by membrane expansion as well as septum material deposition at the division site. Membrane expansion during *S. pombe* cytokinesis is relatively poorly understood. However, there are recent reports indicating that some plasma membrane lipid components become dynamically polarized during the cell cycle. Fluorescence microscopy of live cells after staining with filipin has been used to identify sterol-rich domains in membranes (Wachtler *et al.*, 2003). Filipin is a polyne antibiotic that binds to sterols and it is naturally fluorescent (Wachtler *et al.*, 2003). In interphase cells, sterol-rich domains were observed in the plasma membranes at the growing tips of cells. Following the assembly of the CAR a sterol-rich domain was observed in the plasma membrane at the medial region of the cell and the intensity of Filipin fluorescence at the cell tips was reduced. As the CAR constricted, the invaginating plasma membrane at the division site stained intensely with Filipin. The localization of the sterol-rich domain at the division site was independent of F-actin or the MT cytoskeleton, but dependent on the secretion pathway and on endocytosis (Wachtler *et al.*, 2003; Takeda *et al.*, 2004). This indicates that the sterol-rich domain may be delivered to the distinct plasma membrane for cell growth and cytokinesis possibly through the secretory pathway. The localization of the sterol-rich domain required *S. pombe* Cdc15p, an actomyosin ring component and peripheral plasma membrane protein (Takeda *et al.*, 2004). In cells carrying a *cdc15* ts allele, Filipin fluorescence was observed in a spiral pattern along the length of the cells. Thus *cdc15* may contribute to the organization of sterol-rich domains in the plasma membrane in addition to its role in assembly of the actomyosin ring. The polarized, sterol-rich domains in the plasma membrane may play roles in membrane expansion as well as in the recruitment and/or delivery of target proteins to the division site during cytokinesis (Rajagopalan *et al.*, 2003).

The *S. pombe* cell wall is a network of polysaccharides, glucan and galactomannan (Ishiguro, 1998; Humbel *et al.*, 2001; Matsuo *et al.*, 2004). A typical composition of the cell wall is 42% β-1,3-glucan, between 18% to 28% α-1,3-glucan, between 9% to 14% α-galactomannan, 2% β-1,6-glucan, and a minor proportion of chitin. The distribution of these components within the cell wall was studied by

immunolabeling and transmission electron microscopy. The α-galactomannan coated the cell surface, and the β -1,6-branched β-1,3-glucan and β-1,6-glucan mainly filled the layers of the cell wall under the α-galactomannan. The composition and organization of the septum is distinct from that of the cell wall (Humbel *et al.*, 2001). The septum is composed of a layer of primary septum sandwitched between two layers of secondary septum (Figure 1.3.). The primary septum must be disassembled as the final act of cytokinesis in order to release the two daughter cells. The cell wall, secondary septum and primary septum each contain a β-1,6-branched β-1,3-glucan (Humbel *et al.*, 2001). The cell wall and the secondary septa also contain β-1,6-glucan, a component that is not in the primary septum. The β-1,6-glucan was associated with the Golgi apparatus. It was suggested that this component is synthesized in the endoplasmic reticulum and transported to the cell wall and secondary septum (Humbel *et al.*, 2001). Unique to the primary septum is a linear β-1,3-glucan. This feature suggests that the linear β-1,3-glucan at the primary septum may be required for separation of daughter cells (Humbel *et al.*, 2001).

Several genes were discovered to participate in the synthesis of septum materials. *S. pombe cps1*/*bgs1* (referred to as *cps1* below) encodes one of four β-1,3-glucan synthase catalytic subunits (Cps1, Bgs2p, Bgs3p, and Bgs4p) (Liu *et al.*, 2002; Cortes *et al.*, 2005). The cellular distribution of the *cps1* product has been determined by observing a GFP tagged Cps1p fusion (Cortes *et al.*, 2002; Liu *et al.*, 2002). This fusion protein was found at the growing tip(s) (Cortes *et al.*, 2002) and GFP-Cps1p appeared in the middle of the cell in late anaphase (Cortes *et al.*, 2002; Liu *et al.*, 2002). The medial localization of GFP-Cps1p showed a dramatic change following this (Cortes *et al.*, 2002). The fluorescent signal of this fusion protein first appeared as a faint medial transverse line that joined two bright GFP-Cps1p dots on opposite sides of the cell at the medial cortex. Prior to the constriction of the actomyosin ring, the intensity of the medial line increased. When the actomyosin ring contracted, the medial line also contracted, but a faint fluorescent signal remained as a medial transverse line. Later, when septum formation was complete, the faint line appeared as two, bright bands separated by the septum. These signals persisted but became fainter at the new ends of the two daughter cells as the primary septum was being hydrolyzed. Finally, the GFP-

Cps1p signal disappeared before the two daughter cells were completely separated. The colocalization of GFP-Cps1p with Cdc4p or with a dye associating with β-1,3-glucan was investigated. GFP-Cps1p and β-1,3-glucan were observed at the division plane after Cdc4p was loaded. Cdc4p and GFP-Cps1p contracted at the same time (Liu *et al.*, 2002). These observations suggest that Cps1p is loaded late during actomyosin ring assembly and that it is involved in septum formation during cytokinesis.

To evaluate if the distribution of GFP-Cps1p is dependent on the actomyosin ring and/or the SIN pathway, its localization was investigated in strains carrying mutations affecting components of the actomyosin ring and SIN pathway (Cortes *et al.*, 2002; Liu *et al.*, 2002). The actomyosin ring seems to play a role as a spatial landmark for loading Cps1p in the middle of the cell. The medial localization of Cps1p failed when the assembly of the actomyosin ring was disrupted; whereas, Cps1p localization at the growing cell tips was barely affected. Cps1p medial localization also failed in SIN mutants. The distribution of Cps1p, especially its medial localization, thus requires a functional actomyosin ring and SIN pathway. Furthermore, the Cps1p distribution requires a functional secretory pathway, because the Cps1p localization at the medial plane was not observed when the secretory pathway was disturbed by chemical treatment (Liu *et al.*, 2002).

Interestingly, *cps1* seems to play multiple roles during cytokinesis. As mentioned above, septum formation is one of its roles (Cortes *et al.*, 2002; Liu *et al.*, 2002). The second role may be surveillance for cytokinesis (Le, X *et al.*, 1999; Liu *et al.*, 2000). A phenotype of a *cps1* mutant differed from a typical defective cytokinesis phenotype, which is cell elongation and multiple nuclei. A *cps1* ts mutant was arrested with 2 nuclei and a stable F-actin ring, but neither F-actin ring constriction nor septum formation occurred in this mutant. The 2 nuclei were confirmed to be arrested in G2 (Liu *et al.*, 2000). This result suggested that the G2/M transition might be blocked due to lack of completion of cytokinesis in the *cps1* mutant.

Despite the discovery of some genes involved in septum material synthesis, how the glucans are modified to form the primary and secondary septa, how they are delivered to the division plane, and how *cps1* influences the surveillance system for cytokinesis remain to be investigated.

1.2.2.5. Cell separation

Unlike animal cell division in which plasma membrane ingression and severance at the division site causes the physical separation of two daughter cells, *S. pombe* cell separation is achieved as the last step of cytokinesis by hydrolysis of the primary septum (Figure 1.2.). In *S. pombe*, transmission electron microscopy revealed the structure of the septation area immediately before the hydrolysis of a primary septum occurred (Johnson *et al.*, 1973). There was a dark, triangular shaped area. One side of the triangle contacted the longitudinal cell wall, another side contacted the secondary septum, and the third side was adjacent to the membrane of the daughter cell. This area was refered to as the triangular dense material (Johnson *et al.*, 1973). The triangular dense material was observed at the septation site of both daughter cells. The cell wall area which contacted the triangular dense material was called as a septum edging (Dekker *et al.*, 2004). Cell separation requires the hydrolysis of 3 distinct areas of the septum. They are the middle region of the cell wall adjacent to the septum, the triangular dense material in each daughter cell, and the primary septum (Dekker *et al.*, 2004).

The phenotype associated with defective cell separation in *S. pombe* is an increased septation index or accumulation of chained cells that have undergone one or more rounds of mitosis. Actomyosin ring assembly and constriction, and septum formation at the division site are normal (Wang *et al.*, 2002; Berlin *et al.*, 2003; Wang *et al.*, 2003; Alonso-Nunez *et al.*, 2005; Martin-Cuadrado *et al.*, 2005; Santos *et al.*, 2005).

Cell separation in *S. pombe* appears to be regulated at the level of gene transcription. *S. pombe ace2* and *sep1* encode transcription factors which regulate the transcription of some genes involved in cell separation (Alonso-Nunez *et al.*, 2005). A *sep1* deletion mutant is impaired for *ace2* expression, including transcription of cell separation genes such as *agn1*, *eng1*, and *mid2*. Deletion of *ace2* did not affect *sep1* expression, suggesting that *sep1* probably regulates *ace2* expression (Alonso-Nunez *et al.*, 2005). The *ace2* mRNA level fluctuated in a cell cycle-dependent manner; it peaked before septation. The mRNA levels of cell separation genes also varied during the cell cycle, with the peak levels of their transcripts occuring later in the cycle than the peak level for the *ace2* transcript. The *ace2* deletion mutant failed to express the cell

separation genes, and was defective in cell separation, exhibiting branched chains of cells (Alonso-Nunez *et al.*, 2005).

In fission yeast, defective cell separation phenotypes are produced by mutations in a number of genes. These include: *eng1*, encoding endo-β-1,3-glucanase; *agn1*, encoding endo-α-1,3-glucanase; *rho3* and *rho4*, encoding Rho family GTPases; *mid2*, encoding an anillin homologue; *spn1*, *spn2*, *spn3* and *spn4*, encoding septins; and *sec6*, *sec8*, *sec10*, *sec*15 and *exo70*, encoding exocytosis proteins (exocyst) (Wang *et al.*, 2002; Martin-Cuadrado *et al.*, 2003; Wang *et al.*, 2003; Martin-Cuadrado *et al.*, 2005; Santos *et al.*, 2005). The endo-β-1,3-glucanase Eng1p was reported to be secreted and to act to degrade β-1,3-glucan, resulting in primary septum dissolution (Martin-Cuadrado *et al.*, 2003). Its deletion caused the failure of cell separation due to the persistence of primary septum at the septation site. Eng1p surrounds the septum region as a ring-like structure rather than being coincident with the septum itself (Martin-Cuadrado *et al.*, 2003). Another enzyme, the endo-α-1,3-glucanase Agn1p, was also secreted, it localized to the septum region like Eng1p, and it appears to be involved in cell separation (Dekker *et al.*, 2004). However, an *agn1* deletion mutant phenotype was different from the *eng1* deletion phenotype (Dekker *et al.*, 2004). The *eng1* deletion mutant failed to hydrolyze a primary septum between two new ends of daughter cells. However, the *agn1* mutant partially hydrolyzed the septum edging but the hydrolysis of the primary septum was not affected. The loss-of-function of Agn1p often results in daughter cells still attached at the septum edge. The *S. pombe* cell separation thus seems to be achieved by the distinct enzymatic activities of Eng1p and Agn1p. The question is how these enzymes are delivered to the septation site where they function.

The discovery of the exocyst, an *S. pombe* multiple protein complex functioning in exocytosis, as well as two Rho family GTPases, sheds light on understanding the delivery mechanism for Eng1p and Agn1p (Wang *et al.*, 2002; Wang *et al.*, 2003; Martin-Cuadrado *et al.*, 2005; Santos *et al.*, 2005). The *S. pombe* exocyst is composed of Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p (Wang *et al.*, 2002; Martin-Cuadrado *et al.*, 2005). The phenotype of loss-of-function mutants in these genes was the failure of cell separation with no hydrolysis at the septation site (Wang *et al.*, 2002). Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p are physically associated each other *in vivo*

and they colocalize at regions of active exocytosis, the growing cell ends and the division plane. In particular, the medial localization depends on the actomyosin ring rather than on exocytosis (Wang *et al.*, 2002). Disruption of the actin cytoskeleton or mutations affecting components of the actomyosin ring impaired the localization of Sec10p at the medial plane, but blocking exocytosis by drug treatment did not affect Sec10p medial localization. In *sec8* or *exo70* mutants, both Eng1p and Agn1p failed to localize to the septation site, but their enzyme activities were not affected (Martin-Cuadrado *et al.*, 2005). This suggests that Eng1p and Agn1p are potential cargos for the exocyst (Martin-Cuadrado *et al.*, 2005). Two Rho GTPases, Rho3p and Rho4p, also regulate cell separation (Wang *et al.*, 2003; Santos *et al.*, 2005). The *rho3* gene was identified as a multicopy suppressor of the *sec8* ts mutant (*sec8-1*) since *rho3* overexpression restored the defective phenotype and growth of *sec8-1* (Wang *et al.*, 2003). However, this phenomenon was not observed in a *sec8* deletion mutant, indicating that the *rho3* overexpression seems to suppress the *sec8-1* mutant not through bypassing the Sec8p requirement but through somehow stimulating residual function of the mutant Sec8-1p (Wang *et al.*, 2003). The Rho3p localization at the septation site was dependent on the functional exocyst whereas the exocyst properly localized at the septation site in the absence of Rho3p (Wang *et al.*, 2003). Furthermore, vesicle-like structures were accumulated in the cytoplasm in the *rho3* deletion cells (Wang *et al.*, 2003). These observations taken together suggest that *S. pombe* Rho3p regulates cell separation probably through exocyst function and through regulating secretory vesicle traffic. A *rho4* deletion phenotype was impaired for cell separation and it was rescued by *eng1* or *agn1* overexpression (Santos *et al.*, 2005). The medial localization and secretion of Eng1p or Agn1p were dependent on the presence of Rho4p, especially at higher temperatures, 36-37°C (Santos *et al.*, 2005). Although Rho4p like Rho3p is known to be necessary for exocyst function (Wang *et al.*, 2002; Santos *et al.*, 2003), Rho4p is probably required at the higher temperatures rather than the lower temperatures in order to conduct effective cell separation in *S. pombe*. Thus, depending on the temperature Rho3p and/or Rho4p may contribute to exocyst functions that are required for the proper secretion of the cell separation enzymes Eng1p and Agn1p.

For successful cell separation in *S. pombe*, the enzymatic activities for septum hydrolysis and the correct delivery of the enzymes to the septation site are both required. In addition to these requirements, the structural organization at the septation site is also critical. The cell separation enzymes Eng1p and Agn1p are correctly secreted to the site of cell division in cells carrying deletion alleles of *mid2* or of any of the septin genes; however, the organization of these enzymes at the division site is disrupted, showing a disc-like structure as opposed to the normal ring structure, and cell separation fails to occur (Martin-Cuadrado *et al.*, 2005).

Mid2p and the septins are colocalized in the middle of the cell at late mitosis, forming a ring structure that splits into two rings during septation (Tasto *et al.*, 2003). Loss of Mid2p functions results in disruption of the organization of septin at the septation site, in that septins appear in a disk-shaped structure rather than in a ring. In cells in which *mid2* is overexpressed, septins are observed in misplaced filamentous structures throughout the cell (Tasto *et al.*, 2003).

Although Mid2p is like Mid1p in that it is an anillin-like protein that contains a PH domain, loss of its function does not affect the location, or assembly and contraction of the actomyosin ring, which are functions that are affected by loss of function of Mid1p (Berlin *et al.*, 2003). Interestingly, Mid2p requires its PH-domain for its medial localization and function (Berlin *et al.*, 2003; Tasto *et al.*, 2003), whereas the Mid1p PH-domain is not required for its localization at the division site (Paoletti and Chang, 2000). PH-domains interact with phosphatidylinositol 4-phosphate (PtdIns4P) and/or with other phosphatidylinositol (PtdIns) metabolites, responsible for protein-inositol lipid interactions (De Matteis *et al.*, 2005). The PH-domain of Mid2p is required for both the localization of Mid2p and for cell separation. The PH-domain of Mid2p was not sufficient for either correct localization and for cell separation functions (Berlin *et al.*, 2003; Tasto *et al.*, 2003).

In *S. pombe*, numbers of proteins are identified as having roles in cell separation; Eng1p, Agn1p, Rho3p, Rho4p, Mid2p, the septins, and the exocyst. Cell separation proteins must accumulate at the division site, and assemble into structures, such as the ring structure that contains Mid2p and septins. The disassembly of this ring structure may also be important for regulation of cell separation. Overexpression of a

mid2 allele that encoded an abnormally stable Mid2p protein resulted in persistence of the septin ring and failure of cell separation, suggesting that the degradation of Mid2p is required for these processes (Tasto *et al.*, 2003). The temporal and spatial accumulation and degradation of Mid2p appears to be a key controller for cell separation in *S. pombe*.

1.3. Phosphatidylinositol metabolites in cytokinesis

1.3.1. Overview of metabolism of phosphoinositides

The membrane phospholipid, phosphatidylinositol (PtdIns), is a precursor of several second messengers that regulate cellular processes such as the cell division cycle, cytoskeletal rearrangements and membrane trafficking (Huijbregts *et al.*, 2000; Cockcroft and De Matteis, 2001; Yin and Janmey, 2003; Wong *et al.*, 2005; Emoto *et al.*, 2005; Logan and Mandato, 2006). Phosphoinositides are phosphorylated forms of PtdIns. The general turnover of phosphoinositides is illustrated in Figure 1.4. There are seven metabolites of PtdIns corresponding to the position(s) and number(s) of phosphate groups on the inositol ring. There are three monophosphoinositides, PtdIns 3-phosphate, PtdIns 4-phosphate and PtdIns 5-phosphate (PtdIns3P, PtdIns4P and PtdIns5P); three bisphosphoinositides, PtdIns 3,4-bisphosphate, PtdIns 3,5-bisphosphate and PtdIns 4,5-bisphosphate (PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2); and one trisphosphoinositide, PtdIns 3,4,5-triphosphate (PtdIns(3,4,5)P_3) (Fruman *et al.*, 1998; De Matteis and Godi, 2004). The synthesis and turnover of phosphoinositides are tightly controlled by families of lipid kinases and phosphatases whose localization may allow the formation of discrete pools of phosphoinositides that may have distinct functions (Desrivieres *et al.*, 1998; Audhya *et al.*, 2000; Wera *et al.*, 2001; Foti *et al.*, 2001; Audhya and Emr, 2002; Audhya and Emr, 2003; De Matteis and Godi, 2004; Balla, 2006). The lipid kinases are of three types: PtdIns 3-kinases, PtdIns 4-kinases, and PtdIns-phosphate (PIP) kinases (Fruman *et al.*, 1998). PtdIns 3-kinases phosphorylate at the 3-position of the inositol ring, producing PtdIns3P, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3. PtdIns 4-kinases phosphorylate at the 4-position of the inositol ring, producing only PtdIns4P. PIP kinases phosphorylate at the 4- or 5-position of phosphorylated phosphoinositides, producing only PtdIns(4,5)P_2. In addition, there is a PtdIns(3)P 5-kinase, producing PtdIns(3,5)P_2. PtdIns 3-kinases, PtdIns 4-kinases, or PIP

kinases are further subclassified on the basis on their biochemical features and sequence similarities and on the presence of common domains (Fruman *et al.*, 1998; Lindmo and Stenmark, 2006; Balla and Balla, 2006).

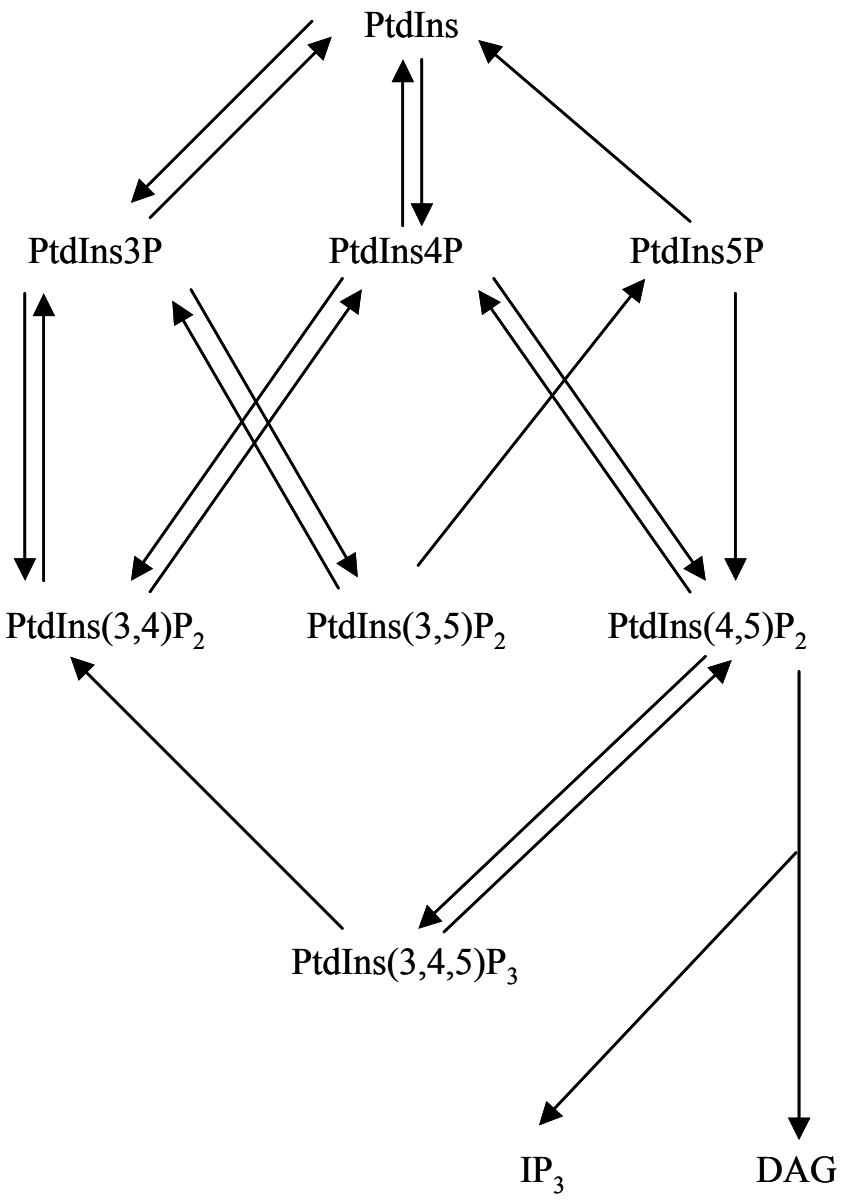


Figure 1.4. Phosphatidylinositol metabolism. There are three PtdIns mono-phosphates (PtdIns3P, PtdIns4P, and PtdIns5P), three PtdIns biphosphates (PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(4,5)P_2), and one PtdIns triphosphate (PtdIns(3,4,5)P_3)as PtdIns derivates. They are produced by actions of various kinases and phosphatases. PtdIns(4,5)P_2 is hydrolysed to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC).

1.3.2. Role(s) of phosphoinositides in cellular functions

PtdIns(4,5)P_2 is the precursor of 2 important secondary messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Figure 1.4.). The initial interest in PtdIns(4,5)P_2 was based on this fact (Pike, 1992). The roles of these two second messengers have been reported. DAG is an intracellular activator for a protein kinase C (PKC) family which is involved in various cellular processes such as the cell cycle, cytoskeleton dynamics, and differentiation (Asaoka *et al.*, 1992; Apgar, 1995). IP_3 stimulates IP_3 receptors in the ER membrane to release Ca^{2+} ions inside cells. These Ca^{2+} ions trigger signal transduction cascades that are involved in various cellular processes (Asaoka *et al.*, 1992; Balla, 2006). In addition, the cloning and functional study of lipid kinases and phosphatases facilitate the understanding of the biological functions of phosphoinositides (Fruman *et al.*, 1998; Balla, 2006; Lindmo and Stenmark, 2006; Balla and Balla, 2006).

The plasma membrane and other intracellular membranes such as the ER and Golgi are dynamic because there are on-going activities like insertion, retrieval, protrusion, budding and fission. It has been speculated that phosphoinositides are localized at discrete membranes; PtdIns3P, PtdIns4P, and PtdIns(3,5)P_2 are predominantly at the intracellular membranes whereas PtdIns(4,5)P_2, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 are mainly at the plasma membrane (Lindmo and Stenmark, 2006; Takenawa and Itoh, 2006). This is supported by the intracellular localizations of their effectors, such as proteins with PH-domains, as well as their generators, the lipid kinases and phosphatases.

In addition to acting as the precursor for DAG and IP_3, PtdIns(4,5)P_2 in the plasma membrane seems to regulate the early stage of clathrin-dependent endocytosis. When a clathrin-coated pit forms, an adaptor complex, called AP-2, which tethers receptors to clathrin becomes localized to the plasma membrane. The AP-2 localization is dependent on its association with PtdIns(4,5)P_2 (Gaidarov and Keen, 1999). To complete the formation of the clathrin-coated vesicle, epsin and the GTPase dynamin, which also bind to PtdIns(4,5)P_2, are recruited. Epsin promotes vesicle formation through its association with clathrin, AP-2 and PtdIns(4,5)P_2, and this results in curvature of the membrane. The binding of PtdIns(4,5)P_2 by GTPase is required for

pinching off of the vesicles from the plasma membrane (Hinshaw and Schmid, 1995; Takei *et al.*, 1995; Ford *et al.*, 2002). The PtdIns(4,5)P_2 in these endocytic vessicles is converted to PtdIns by the activity of synaptojanin. This results in release of clathrin (Cremona *et al.*, 1999). The vessicles then become enriched with PtdIns3P as the result of the activity of the type III PtdIns 3-kinase which uses PtdIns as its substrate (Lindmo and Stenmark, 2006). The endocytic vesicle is then fused to the endosome. This fusion is achieved through proteins that specifically bind PtdIns3P (Simonsen *et al.*, 1998; Nielsen *et al.*, 2000; Schnatwinkel *et al.*, 2004). The early endosome can be recycled or can become the late endosome which then fuses with lysosomes or vacuoles for degradative trafficking in cells. The early endosome moves along MTs either toward the plus-end or the minus-end depending on which motor protein it associates with. This mobility seems to be dependent on PtdIns3P (Hoepfner *et al.*, 2005).

In *S. cerevisiae* cells, loss-of-function of a PtdIns3P 5-kinase, Fab1p, resulted in impaired vacuole morphology and function (Yamamoto *et al.*, 1995; McEwen *et al.*, 1999; Odorizzi *et al.*, 2000; Onishi *et al.*, 2003). It also resulted in failure of the degradation pathway for some cargoes of the endosomes (Odorizzi *et al.*, 1998). This indicates that the conversion of PtdIns3P to PtdIns(3,5)P_2 by the PtdIns3P 5-kinase results in the early endosome becoming a late endosome that is targeted for the degradative pathway.

PtdIns(3,4,5)P_3 is present at the plasma membrane although at low levels (Toker and Cantley, 1997). It is mainly generated by type I PtdIns 3-kinases, which phosphorylate PtdIns(4,5)P_2 *in vivo* (Lindmo and Stenmark, 2006). PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 have been reported to be required for a stage of phagocytosis, phagocytic cup sealing, which produces a phagosome in animal cell lines (Marshall *et al.*, 2001). A fusion gene encoding GFP fused to a protein domain that is specific for phosphoinositides that are phosphorylated at the 3-position was created. A plasmid expressing this gene was transfected into a cell line. The GFP fluorescence signal was detected at the phagocytic cup, and it immediately disappeared as the cup was sealed. This indicates that the level of PtdIns(3,4,5)P_3 has to be precisely regulated for effective phagocytosis. Consistent with this suggestion, the type I PtdIns 3-kinase was present in

the phagocytic cup membrane. Recent studies suggest that PtdIns(3,4,5)P_3 may be involved in cytokinesis. This subject is examined separately in section 1.3.2.1.

PtdIns4P seems to be required for the secretory pathway. A conditional allele of *S. cerevisiae PIK1*, which encodes a type IIIβ PtdIns 4-kinase, did not produce PtdIns4P at the restrictive temperature and was defective for secretion (Walch-Solimena and Novick, 1999; Hama *et al.*, 1999; Audhya *et al.*, 2000). Although there are several PtdIns 4-kinase paralogues in many organisms, their only substrate is PtdIns (Balla, 1998; Balla and Balla, 2006). The localizations and functions of PtdIns 4-kinases and of their product, PtdIns4P will be discussed in section 1.3.3.

A variety of actin-binding proteins have been reported to contain phosphoinositide-binding domains with specificities for various phosphoinositides (Sechi and Wehland, 2000; Yin and Janmey, 2003). In particular, PtdIns(4,5)P_2 appears to be significant for actin cytoskeleton dynamics such as polymerization and depolymerization. In general, high levels of PtdIns(4,5)P_2 stimulate actin polymerization whereas low levels trigger disassembly. For example, neuronal Wiskott-Aldrich syndrome protein (N-WASp) is activated when it binds both PtdIns(4,5)P_2 and a small GTPase, Cdc42. Activated N-WASp then induces F-actin polymerization (Rohatgi *et al.*, 1999). Actin severing proteins such as gelsolin and cofilin/ADF (actin depolymerizing factor) are inactivated by their association with PtdIns(4,5)P_2 (Janmey and Stossel, 1987; Yonezawa *et al.*, 1990). Also, PtdIns(4,5)P_2 mediates anchoring of the actin cytoskeleton to the plasma membrane through modulating some other actin-binding proteins (Sechi and Wehland, 2000). The actin cytoskeleton dynamics mediated by PtdIns(4,5)P_2 result in cellular conformational changes, and participate in processes such as plasma membrane attachment and focal adhesion, and they are involved in many processes of the cell cycle (Logan and Mandato, 2006).

In summary, specific phosphoinositides localize within specific membranes in order to regulate a variety of cellular processes. The distribution within the cell of the lipid kinases and phosphatases that control the levels of each specific phosphoinositide and of the effector proteins that bind them, reflect this fact.

1.3.2.1. Phosphoinositides and cytokinesis

The results of studies in diverse organisms indicate that several phosphoinositides have roles during cytokinesis. In animal cells, following the assembly of the actomyosin ring, cleavage furrow ingression takes place at the division site as the actomyosin ring constricts. The injection of anti-PtdIns(4,5)P_2 antibodies into *Xenopus* oocytes arrested the cell division cycle (Han *et al.*, 1992). This treatment might be expected to reduce the free pool of PtdIns(4,5)P_2 and this reduction in itself might have had a direct effect. This reduction would also be expected to reduce the levels of DAG and IP_3, which are produced from PtdIns(4,5)P_2 by the action of phospholipase C. Reduced levels of DAG and IP_3 might be involved in the arrest of cleavage furrow ingression. Early experiments involving measurements of levels of DAG and Ca^{2+}, and the use of inhibitors of IP_3 receptors and chelators of Ca^{2+} failed to fully resolve these questions (Han *et al.*, 1992; Miller *et al.*, 1993; Lee *et al.*, 2003). It has been proposed that PtdIns(4,5)P_2 itself, in addition to Ca^{2+}, might be required for normal progression of cytokinesis (Wong *et al.*, 2005). The PH-domain from a human PLCδ enzyme specifically binds to PtdIns(4,5)P_2 (Harlan *et al.*, 1994). A gene encoding this domain fused to GFP was expressed in *Drosophila* cells and the fluorescent signal was found at the plasma membrane and cleavage furrow of dividing cells (Field *et al.*, 2005). It is assumed that the PH-domain is localizing the fusion protein to sites where PtdIns(4,5)P_2 is relatively concentrated. The function of PtdIns(4,5)P_2 at the cleavage furrow is uncertain. One potential function proposed is that PtdIns(4,5)P_2 plays a role in the adhesion of the actomyosin ring to the plasma membrane (Field *et al.*, 2005). In addition to the use of PH-domains to identify the distribution of PtdIns(4,5)P_2, they can be used to sequester this molecule by expressing the fusion gene at higher levels. In such a study in several mammalian cell lines, overexpression of a gene encoding the PH-domain from PLCδ fused to an enhanced GFP resulted in the separation of the F-actin ring from the plasma membrane at the furrow and in multinucleated cells (Field *et al.*, 2005).

The other proposed function for PtdIns(4,5)P_2 is to suppress actin filament bundling at the cleavage furrow via negatively regulating the actin-bundling protein, cortexillin I (Stock *et al.*, 1999). Cortexillin I was identified in *D. discoidium* as an actin-bundling protein that is recruited to the cleavage furrow cortex (Weber *et al.*, 1999). It binds to actin and is implicated in cytokinesis (Weber *et al.*, 1999). Cortexillin I is

composed of an N-terminal actin-binding domain, a central region responsible for the formation of a parallel two-stranded coiled coil, and a C-terminal domain including basic residues that are reminiscent of a PtdIns(4,5)P_2-binding motif (Weber *et al.*, 1999). This latter domain was shown to bind to PtdIns(4,5)P_2 (Stock *et al.*, 1999). The proportion of multinucleate cells increased when cells carried a cortexillin I deletion allele. Expression of a gene encoding the C-terminal domain of cortexillin I was sufficient to restore this cytokinesis defect; whereas, the N-terminal was not (Stock *et al.*, 1999; Weber *et al.*, 1999). In addition, a GFP-fused C-terminal domain was localized to the cleavage furrow cortex whereas a GFP-fused N-terminal domain was dispersed throughout the cytoplasm (Weber *et al.*, 1999). However, the PtdIns(4,5)P_2-binding motif at the C-terminal end was not required for the localization of the GFP-fused C-terminal domain to the cleavage furrow cortex (Stock *et al.*, 1999). Rather, this motif was required for the prevention of actin-bundling in the presence of PtdIns(4,5)P_2 (Stock *et al.*, 1999). Cortexillin I inhibited actin bundling in the presence of PtdIns(4,5)P_2, but not in the absence of PtdIns(4,5)P_2. This suggests that PtdIns(4,5)P_2 is required for the regulation of actin bundling at the cleavage furrow in dividing cells.

Based on studies of PtdIns(4,5)P_2 in diverse organisms, several questions are raised: How is the PtdIns(4,5)P_2 level regulated at the division site during cytokinesis? Is PtdIns(4,5)P_2 synthesized through the same or different metabolic pathways at the division site in different organisms? Although most answers are uncertain yet, some investigations give clues about these questions.

First, the *S. pombe* phosphoinositide phosphatase, Ptn1p, which is specific for the 3-position of the inositol ring, was localized in the cytoplasm with a punctate pattern, or at the division plane during cytokinesis. Cells carrying a *ptn*1 deletion allele had increased levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, indicating that these phosphoinositides may be substrates for Ptn1p. Furthermore, PtdIns(3,4,5)P_3 was not detected in wild-type *S. pombe* cells whereas it seemed to appear at the division site in the *ptn*1 deletion mutant (Mitra *et al.*, 2004). These observations were consistent with those made in *D. discoideum* regarding PTEN, which is homologous to Ptn1p (Janetopoulos *et al.*, 2005). The distribution of PTEN was restricted to the cleavage furrow cortex. This suggests that the phosphatase PTEN is localized to the division site

in order to produce PtdIns(4,5)P_2 and/or PtdIns4P, which presumably have a role during cytokinesis. In the case of *D. discoideum*, phosphoinositide metabolism and signaling seem to be spatially regulated during cytokinesis. Secondly, the *Drosophila four-wheel-drive* (*fwd*) gene encodes a PtdIns 4-kinase (Brill *et al.*, 2000). Mutants in this gene are defective in cytokinesis; the actin ring assembles and constricts, but cleavage furrow ingression and separation fail, resulting in multinucleated cells. Although the level of PtdIns4P was not measured, the authors proposed that *Drosophila* PtdIns 4-kinase regulates late cytokinesis probably via supplying the substrate PtdIns4P for production of PtdIns(4,5)P_2. PtdIns4P 5-kinases convert PtdIns4P to PtdIns(4,5)P_2 (Takenawa and Itoh, 2001; Yin and Janmey, 2003). It was reported that mammalian and *S. pombe* PtdIns4P 5-kinases, PIP5Kβ and Its3p, respectively, were localized at the division site (Emoto *et al.*, 2005; Balla, 2006). The results from both of these studies suggested that the PtdIns4P 5-kinases are required for regulating the level of PtdIns(4,5)P_2 during cytokinesis. Indeed, in *S. pombe* cells carrying a conditional allele of *its3*, levels of PtdIns(4,5)P_2 were severely decreased and levels of PtdIns4P were increased at the restrictive temperature (Zhang *et al.*, 2000). Furthermore, this mutant increased the septation index at the restrictive temperature, which is characteristic of a cytokinesis defect (Zhang *et al.*, 2000). In mammalian cells, a PIP5Kβ mutant that was defective for kinase activity failed to localize correctly to the cleavage furrow and the cells became multinucleate (Emoto *et al.*, 2005). This suggests that in these systems, cytokinesis normally involves the localization of PtdIns4P 5-kinases to the division site where they produce PtdIns(4,5)P_2 from PtdIns4P.

Thus, PtdIns(4,5)P_2 was found to be required at the division site for cytokinesis in *S. pombe*, *D. discoideum*, *Drosophila*, and mammals. In the case of *S. pombe*, this phosphoinositide seems to be produced at the division site by the actions of both a phosphatase on PtdIns(3,4,5)P_3 and of a PtdIns4P 5-kinase on PtdIns4P. Whether these actions occur simultaneously during cytokinesis and whether they occur in the same compartment at the division site have to be investigated. The other question to be addressed would be whether both of these activities are also present during cytokinesis in *D. discoideum*, *Drosophila*, and mammals.

1.3.3. Phosphatidylinositol 4-kinases

1.3.3.1. Biochemical features and classification

Among the lipid kinases, PtdIns 4-kinases (EC 2.7.1.67) convert PtdIns to PtdIns4P by phosphorylating the 4-position of the inositol ring. PtdIns4P is a precursor for several phosphoinositides (Figure 1.4.). PtdIns 4-kinases have been classified into 4 types, Type IIα, Type IIβ, Type IIIα and Type IIIβ, on the basis of biochemical studies as well as sequence similarities (Balla, 1998; Balla and Balla, 2006). Table 1.2. summarizes the biochemical features and lists orthologues that have been identified in various organisms.

Type II PtdIns 4-kinases appear to be membrane associated in mammalian cells (Pike, 1992). Further characterization indicated that these PtdIns 4-kinases in the membrane fraction were sensitive to inhibition by adenosine, a characteristic which distinguishes them from the type III family PtdIns 4-kinases. In addition, the type II PtdIns 4-kinases localize to the membrane by palmitoylation (Barylko *et al.*, 2001). Two subfamilies, the α-isoforms and β-isoforms, exist in the type II family (Gehrmann and Heilmeyer, Jr., 1998; Balla and Balla, 2006). The biochemical characteristics of the members of these two subfamilies are very similar, even their molecular weights (Table 1.2.). They are not sensitive to the inhibitors of PtdIns 3-kinases, wortmannin and LY 294002, and they are inhibited by Ca^{2+}.

There are also two subfamilies of the type III PtdIns 4-kinases, the α- and β-isoforms. The general biochemical features of these are similar, but their molecular weights are different, indicating that they probably have unique feature(s) of their domain structures. Indeed, the majority of type III PtdIns 4-kinase α-isoform proteins identified from diverse organisms contain a PH domain adjacent to their C-terminal catalytic domain and they possess relatively long N-terminal domains. The β-isoforms do not contain a PH domain and they have shorter N-terminal domains (Gehrmann and Heilmeyer, Jr., 1998; Heilmeyer, Jr. *et al.*, 2003; Balla and Balla, 2006). Unlike the type II PtdIns 4-kinases, Ca^{2+} has no effect on the type III PtdIns 4-kinases, and the type III isoforms have a lower affinity for their substrate, PtdIns, than that of the type II isoforms.

Table 1.2. Characterizations and Properties of PtdIns 4-kinase Enzymes

Classes	Type IIα	Type IIβ	Type IIIα	Type IIIβ	
					Organism
Names	SPAC343.19		SPBC577.06c	Pik1p (851)	*S. pombe*
	Lsb6p (607)		Stt4p (1900)	Pik1p (1066)	*S. cerevisiae*
				fwd PI4K (1338)	*Drosophila*
				DdPIK4 (1093)	*D. discoideum*
	hPI4KIIα (479)	hPI4KIIβ (481)	hPI4K230 (2044)	hPI4Kβ (801), hNPIK-A (828), hNPIK-C (816)	human
			bPI4KIIIα (2043)	bPI4KIIIβ (801)	bovine
	rPI4KII (478)		rPI4K230 (2041)	rPI4K92 (816)	rat
	mPI4KII (479)	mPI4KIIβ (469)			mouse
					Property
Characteristics	55-56	55-56	210	110	Molecular weight (kDa)
	Insensitive	Insensitive	Sensitive	Sensitive	Wortmannin
	Insensitive	Insensitive	Sensitive	Sensitive	LY 294002
	Inhibits	Inhibits	No direct effect	No direct effect	Ca^{2+}-sensitivity
	10-70 μM	10-70 μM	Millimolar	Millimolar	Ki (adenosine)
	Activates	Activates	Activates	Activates	Triton X-100
	10-50 μM	10-50 μM	~700 μM	~400 μM	K_m (ATP)
	~20-60 μM	~20-60 μM	~100 μM	~100 μM	K_m (PtdIns)

(Numbers in brackets are amino acid numbers.)

1.3.3.2. Localization of PtdIns 4-kinases and postulated functions

As indicated in Table 1.2., diverse organisms possess several PtdIns 4-kinases that each produce the same product, PtdIns4P. The functions of some paralogues are known to be non-redundant. Why multiple enzymes are required to produce the same product, PtdIns4P, and how the product from each isoform is recognized separately by different effectors are still unclear. However, studies in different organisms suggest that the answer may be found in distinct patterns of distribution within the cell for each isoform. The following discussion is focused on the three PtdIns 4-kinases in *S. cerevisiae* with some information regarding their orthologues in other eukaryotes.

Three PtdIns 4-kinases have been identified in *S. cerevisiae*. Pik1p is a type IIIβ PtdIns 4-kinase (Flanagan *et al.*, 1993). Stt4p is a type IIIα PtdIns 4-kinase (Yoshida *et al.*, 1994). Lsb6p is a type IIα PtdIns 4-kinase (Han *et al.*, 2002; Shelton *et al.*, 2003). Pik1p and Stt4p are each essential for cell viability, whereas Lsb6p is dispensable. Apparently, Pik1p and Stt4p each have essential functions that are not redundant. Lsb6p has been reported to localize in vacuolar membranes as well as in the plasma membrane (Han *et al.*, 2002; Shelton *et al.*, 2003), Stt4p is localized in the plasma membrane (Audhya and Emr, 2002), and Pik1p resides in Golgi and in the nucleus (Garcia-Bustos *et al.*, 1994; Walch-Solimena and Novick, 1999; Strahl *et al.*, 2005).

Lsb6p has been reported to have a role in the regulation of actin cytoskeleton dynamics during endocytosis (Chang *et al.*, 2005). During endocytosis, endosomes that pinch off from the plasma membrane seem to be transported away from the membrane by the action of actin polymerization (Chang *et al.*, 2005). Lsb6p was identified and characterized in yeast two-hybrid assays as a partner interacting with the *S. cerevisiae* Wiskott-Aldrich Syndrome protein (WASP) orthologue, Las17p, (Madania *et al.*, 1999). The interaction of Lsb6p with Las17p activates the Arp2/3 complex. The activated Arp2/3 complex then promotes the actin polymerization that is required for endosome mobility (Madania *et al.*, 1999; Chang *et al.*, 2005). Arp2p and Arp3p are members of a family of actin-related proteins that are localized at actin cortical patches (Morrell *et al.*, 1999). Interestingly, Lsb6p does not require its PtdIns 4-kinase activity for its function as an activator of Las17p (Chang *et al.*, 2005). A truncated version of Lsb6p that lacked PtdIns 4-kinase catalytic activity was able to interact with Las17p. Furthermore,

expression of the gene encoding this truncated version of Lsb6p rescued the endosome motility defect of cells carrying a deletion allele of *LSB6*. Thus, functions of Lsb6p that lie outside of the lipid kinase catalytic domain are responsible for the interaction with Las17p and for endosome mobility (Chang *et al.*, 2005). However, it is still unclear where, when, or how the PtdIns4P pool produced by Lsb6p activity is utilized in *S. cerevisiae*.

S. cerevisiae Stt4p produces PtdIns4P at the plasma membrane that is involved in the regulation of actin cytoskeleton organization, cell wall integrity and the maintenance of vacuole morphology (Audhya *et al.*, 2000; Foti *et al.*, 2001; Audhya and Emr, 2002). In addition, Stt4p has been proposed to be involved in a checkpoint that delays the cell division cycle when the positioning of the mitotic spindle is perturbed (Muhua *et al.*, 1998). This delay is abolished in cells carrying a mutant allele of *STT4* (*stt4-7*), however the mechanism remains unknown. Cells carrying a conditional *stt4* allele (*stt4-4*) had aberrant vacuole morphology at the restrictive temperature (Audhya *et al.*, 2000; Foti *et al.*, 2001). Cells carrying this conditional allele had reduced levels of PtdIns4P and PtdIns(4,5)P_2 at the restrictive temperature and the organization of the actin cytoskeleton was disrupted in these cells (Audhya *et al.*, 2000). A similar phenotype was observed in cells carrying a conditional allele of the *MSS4* gene (*mss4-2*) (Audhya *et al.*, 2000). *MSS4* encodes a PtdIns4P 5-kinase that is present at the plasma membrane (Audhya and Emr, 2002). The plasma membrane localization of both Stt4p and Mss4p is crucial for PtdIns(4,5)P_2 production that is required for the activation of the Rho1p GTPase (Audhya and Emr, 2002). The Stt4p-dependent PtdIns(4,5)P_2 recruits the guanine nucleotide exchange factor (GEF), Rom2p, to the plasma membrane *via* interacting with a Rom2p PH domain. Rom2p activates the Rho1p GTPase and subsequently Rho1p activates the *S. cerevisiae* protein kinase C, Pkc1p, that is required for cell wall integrity (Levin, 2005). The Rom2p localization to the plasma membrane was disrupted in cells carrying the conditional alleles *stt4-4* or *mss4-102*, but not in cells carrying the conditional allele *pik1-83* at the restrictive temperature (Audhya and Emr, 2002). The role of Stt4p as an activator of Rom2p is important because this appears to be the only pathway for activation of Pkc1p. In contrast to members of the classical PKC family, *S. cerevisiae* Pkc1p is not stimulated by DAG and Ca^{2+} (Levin, 2005). Thus, the Stt4p-

dependent PtdIns4P pool may be required for activation of Pkc1p in *S. cerevisiae*. Although the disruption of actin structure was observed in *S. cerevisiae* cells carrying a conditional *stt4* allele, the mechanism by which Stt4p regulates the organization of the actin cytoskeleton is still uncertain. In contrast to the *S. cerevisiae* Stt4p, mammalian type IIIα PtdIns 4-kinases are found predominantly in the ER (Wong *et al.*, 1997). The *Arabidopsis* orthologue, AtPI4Kα1, is also predominantly found in the ER (Stevenson-Paulik *et al.*, 2003). Whereas the role of the mammalian protein in the ER is not yet known, AtPI4Kα1 is proposed to regulate actin structure (Stevenson-Paulik *et al.*, 2003). The PH domain of AtPI4Kα1 associated with actin filaments *in vitro*. Although *S. cerevisiae* Stt4p mainly localizes at the plasma membrane, it also seems to have functions in the ER (Foti *et al.*, 2001). The *S. cerevisiae* lipid phosphatase, Sac1p, which is localized in the ER, dephosphorylates the Stt4p-dependent PtdIns4P pool, but not the Pik1p-dependent PtdIns4P pool. The localization of Sac1p to the ER appears to be crucial for turnover of PtdIns4P because cells carrying an allele of *SAC1* that encoded active, but mislocalized Sac1p, accumulated PtdIns4P. The loss of Sac1p function affects normal vacuole morphology, similar to the effect of loss-of-function of Stt4p (Foti *et al.*, 2001). These observations demonstrate that the localization of lipid kinases and phosphatases are important for the regulation of levels of local phosphoinositides.

As discussed above, in *S. cerevisiae*, the production of PtdIns4P is important for the regulation of level of PtdIns(4,5)P_2. Also as discussed above, the PtdIns4P produced by the *S. cerevisiae* type IIIβ PtdIns 4-kinase, Pik1p, is itself involved in various cellular processes, especially the Golgi-driven secretory pathway (Garcia-Bustos *et al.*, 1994; Walch-Solimena and Novick, 1999; Hama *et al.*, 1999; Audhya *et al.*, 2000; Nguyen *et al.*, 2005; Strahl *et al.*, 2005). Pik1p localizes to and generates the PtdIns4P pool in the Golgi, and the Pik1p-dependent PtdIns4P pool does not seem to be converted to PtdIns(4,5)P_2 because the only PtdIns4P 5-kinase activity in *S. cerevisiae*, that of Mss4p, was detected at the plasma membrane but not in the Golgi (Walch-Solimena and Novick, 1999; Audhya and Emr, 2003; Strahl *et al.*, 2005). The localization of Pik1p to the Golgi, and its activity, are both essential for its functions (Strahl *et al.*, 2005). In cells carrying a conditional allele of *PIK1*, protein secretion was blocked in the Golgi, indicating that Pik1p is involved in the late stage of normal protein

secretion to the plasma membrane (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000; Nguyen *et al.*, 2005). This is supported by observations regarding the localization of Pik1p and by genetic interactions between *PIK1* and other genes. Pik1p was co-localized with a *trans*-Golgi marker protein but not with a *cis*-Golgi marker protein. *PIK1* genetically interacts with *YPT31*, which encodes a Golgi-associated Rab-GTPase that is required for the Golgi secretory function (Sciorra *et al.*, 2005). *PIK1* also genetically interacts with three *S. cerevisiae SJL* genes (*SJL1*, *SJL2*, and *SJL3*), which encode homologues of the mammalian synaptic vesicle-associated PtdIns(4,5)P_2 5-phosphatase, synaptojanin (Nguyen *et al.*, 2005). *S. cerevisiae* Sjl1p, Sjl2p, and Sjl3p are required for membrane traffic (Nguyen *et al.*, 2005). A double mutant carrying a *pik1* ts allele and a *YPT31* deletion allele was synthetically lethal at a semi-restrictive temperature and displayed defective protein secretion and/or recycling even at a permissive temperature (Sciorra *et al.*, 2005). Cells carrying a conditional *pik1* allele and a deletion allele of *SJL1*, *SJL2* or *SJL3* had decreased levels of PtdIns4P but increased levels of PtdIns(4,5)P_2 at the restrictive temperature. The *pik1*^{ts}/ Δ*sjl3* double mutant was synthetically lethal, and the *pik1*^{ts}/ Δ*sjl1* or *pik1*^{ts}/ Δ*sjl2* double mutants exacerbated the protein secretion defect at a semi-restrictive temperature (34ºC). Pik1p also seems to regulate protein sorting from the Golgi to vacuoles (Walch-Solimena and Novick, 1999; Nguyen *et al.*, 2005). In cells carrying a conditional *PIK1* allele, a labeled protein that is normally sorted into vacuoles through the ER-to-Golgi traffic, accumulated in the Golgi. Pik1p thus may regulate effective protein sorting into vacuoles. Pik1p functions are also required for the maintenance of Golgi structures as examined by transmission electron microscopy (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000; Strahl *et al.*, 2005). Whether a plasma membrane protein (Ste6p) was effectively endocytosed and targeted into vacuoles for its degradation were investigated in cells carrying a conditional *PIK1* allele. This protein failed to be degraded in the *pik1* ts mutant at the restrictive temperature whereas this process was not affected in wild-type cells or in the *stt4* ts mutant at the restrictive temperature. In addition, the uptake of a vital dye, FM4-64, to visualize the endocytic compartments was blocked in the endosomes in the *pik1* ts mutant at the restrictive temperature (Walch-Solimena and Novick, 1999). This indicates that Pik1p is required for effective endocytosis.

Study of *PIK1* alleles that were defective for localization of the protein to the nucleus or to the Golgi demonstrated that Pik1p functions in the nucleus are essential in addition to its functions in the Golgi (Strahl *et al.*, 2005). A diploid strain hemizygous for the *PIK1* locus, *pik1*::*Kan*^{R}/*PIK1*, was transformed with a plasmid that expressed a *pik1* allele that was defective for nuclear localization, or an allele that was defective for Golgi localization. A third strain was transformed with both plasmids. Each strain was allowed to go through meiosis and tetrad dissection was performed. In the cases where only 1 plasmid was present, only 2 of 4 spores formed colonies and those colonies carried the intact *PIK1* locus. In the case of the strain that carried both plasmids, all 4 spores formed colonies. Two colonies were identified that contained the *pik1*::*Kan*^{R} locus and both episomes for further study. Each localization mutant possessed PtdIns 4-kinase catalytic activity, which was determined by *in vitro* lipid kinase activity analysis. Thus, both mutant alleles must be expressed to provide all of the essential functions of Pik1p. A similar experiment was performed with *pik1* alleles that were defective for catalytic activity and for localization. The results demonstrated that Pik1p activity in both the nucleus and the Golgi is essential for cell viability.

The nuclear localization of Pik1p requires that it interacts with the β-importin, Kap95p, through its N-terminal domain. Furthermore, its nuclear export depends on a functional exportin, Msn5p, suggesting that Pik1p shuttles between the nucleus and the cytoplasm in *S. cerevisiae* (Strahl *et al.*, 2005). Surprisingly, Mss4p, which in the plasma membrane uses PtdIns4P that is produced by Stt4p, also shuttles between the nucleus and the cytoplasm (Audhya and Emr, 2003). It is not known if Stt4p also performs this shuttle; however, it has been proposed that Pik1p provides the precursor PtdIns4P pool for Mss4p in the nucleus (Strahl *et al.*, 2005; Balla and Balla, 2006). The functions of these phosphoinositides in the nucleus remain to be determined.

The function of type IIIβ PtdIns 4-kinases in the Golgi seems to be conserved. Mammalian and *Arabidopsis* orthologues are also dominantly localized in the Golgi (Wong *et al.*, 1997; Godi *et al.*, 1999; Preuss *et al.*, 2006). Recruitment of the mammalian type IIIβ PtdIns 4-kinase to the Golgi requires a small GTPase ADP-ribosylation factor (ARF). The mammalian ARF maintains Golgi structure and regulates Golgi functions, especially membrane traffic from Golgi to plasma membrane in the

coordination with the four-phosphate-adaptor proteins (FAPP1 and FAPP2) (Godi *et al.*, 2004). The mammalian type IIIβ PtdIns 4-kinase activity is required in the Golgi for the structural integrity of the Golgi complex. The evidence for this is that a kinase activity-null allele failed to restore Golgi structures disrupted by drug treatment/wash-out (Godi *et al.*, 1999). Although the role(s) of the mammalian type IIIβ PtdIns 4-kinase in Gogi-driven traffic is speculated, the molecular mechanism remains obscure. Like *S. cerevisiae* Pik1p, the mammalian type IIIβ PtdIns 4-kinase is also localized in the nucleus (de Graaf *et al.*, 2002), but its role is uncertain in present.

The type IIIβ PtdIns 4-kinase orthologues in *S. cerevisiae* (*PIK1*), and in *Drosophila* (*fwd*) seem to play a significant role during cytokinesis (Brill *et al.*, 2000; de Graaf *et al.*, 2002). *S. cerevisiae* cells carrying conditional alleles of *pik1* became enlarged and multinucleate (*pik1-101*), or one or two daughter cells remained attached to the mother cell (*pik1-83*) (Garcia-Bustos *et al.*, 1994; Walch-Solimena and Novick, 1999). However, the specific aspects of cytokinesis that require Pik1p activity is uncertain. Two observations are suggestive (Walch-Solimena and Novick, 1999). In the case of both *pik1-101* and *pik1-83* the defective protein failed to produce PtdIns4P at the restrictive temperature. In *pik1-101* cells, F-actin patches were dispersed throughout cytoplasm instead of being polarized during cytokinesis. This indicates that Pik1p catalytic activity is required for proper cytokinesis probably through regulating actin cytoskeleton dynamics. A *Drosophila fwd* mutant was able to assemble and constrict the actomyosin ring, and to initiate the cleavage furrow in dividing spermatocytes. However, the cleavage furrow was unstable and its regression resulted in fusion of the daughter cells and the formation of multinucleated cells (Brill *et al.*, 2000). This suggests that the *Drosophila fwd* PtdIns 4-kinase may be required for the maintenance of the cleavage furrow or for its ingression. However, its mechanism remains uncertain.

In summary, PtdIns 4-kinases play important roles in several biological processes through their catalytic activities which produce PtdIns4P pools and/or their abilities to physically interact with other proteins. The PtdIns4P pools seem to be discrete because each PtdIns 4-kinase normally resides in a distinct subcellular location.

1.3.3.3. Regulation of expression and activity of PtdIns 4-kinases

How the expression of genes encoding PtdIns 4-kinases are regulated and how the activities of the proteins are regulated are not clearly understood. Some studies suggest potential regulatory mechanisms. *S. cerevisiae* Pik1p physically interacts with a calcium-binding protein, frequenin (Frq1p) (Hendricks *et al.*, 1999; Huttner *et al.*, 2003). Frq1p binds Ca^{2+}, is N-myristoylated, and is essential for cell viability (Hendricks *et al.*, 1999). Overexpression of *PIK1* rescues the growth defects of a *frq1* deletion allele or of a conditional allele at the restrictive temperature; whereas, *FRQ1* overexpression fails to rescue the growth defect of a *PIK1* deletion mutant (Hendricks *et al.*, 1999). This indicates Pik1p acts downstream of Frq1p. Pik1p catalytic activity is positively regulated by Frq1p (Hendricks *et al.*, 1999). The essential role(s) of frequenin seems to be conserved; a human orthologue, neuronal calcium sensor-1 (NCS-1), complements the lethality of an *S. cerevisiae FRQ1* deletion mutant (Strahl *et al.*, 2003). NCS-1 also physically interacts with a mammalian type IIIβ PtdIns 4-kinase and regulates its catalytic activity (Zhao *et al.*, 2001). One possible role has been proposed; NCS-1, the type IIIβ PtdIns 4-kinase and ARF may act in concert to play a role in Golgi-to-plasma membrane traffic (Haynes *et al.*, 2005). However, although the relationship of frequenin and type IIIβ PtdIns 4-kinases appears to be conserved in both *S. cerevisiae* and mammalian systems, the roles of its interaction is not fully understood yet.

1.4. Hypothesis and Objectives

1.4.1. Hypothesis: The phosphatidylinositol 4-kinase, Pik1p, is required for cytokinesis in *S. pombe*

In our laboratory previously, structural and genetic studies of *S. pombe* Cdc4p were conducted (Slupsky *et al.*, 2001; Desautels *et al.*, 2001). A small EF-hand protein, Cdc4p has been reported to be a myosin II essential light chain (ELC) and to be an indispensable actomyosin ring component (McCollum *et al.*, 1995). Although Cdc4p is classified as an EF-hand protein, it does not bind Ca^{2+}, at least *in vitro* (McCollum *et al.*, 1995; Slupsky *et al.*, 2001). Cdc4p associates with the myosin II heavy chains, Myo2p and Myp2p, at IQ-domains on the neck regions of these proteins (D'souza *et al.*, 2001). However, several lines of genetic and structural evidence suggest that Cdc4p may have multiple functions in addition to the role of ELC (Desautels *et al.*, 2001; Lord and

Pollard, 2004). Cdc4p is structurally composed of N-terminal and C-terminal domains which include two EF-hands in each domain, and a flexible linker connecting these two domains (Slupsky *et al.*, 2001). Several *cdc4* ts mutants have been identified: *cdc4*^{F12L}, *cdc4*^{G19F} and *cdc4*^{R33K} in the N-terminal domain; *cdc4*^{G82D} and *cdc4*^{G107S} in the C-terminal domain (Nurse *et al.*, 1976; McCollum *et al.*, 1995; Desautels *et al.*, 2001). These ts mutants are viable at the permissive temperature, but not at the restrictive temperature. However, these single *cdc4* ts alleles display genetic complexity (Slupsky *et al.*, 2001; Desautels *et al.*, 2001). Heterozygous diploid cells carrying different combinations of the mutant alleles are viable at the restrictive temperature. At 36^{o}C, the following heterozygous diploids are viable whereas each homozygous diploid is not viable: *cdc4*^{F12L}/*cdc4*^{R33K}, *cdc4*^{F12L}/ *cdc4*^{G82D}, and *cdc4*^{G19F}/*cdc4*^{G107S}. Interallelic complementation occurs in multimeric proteins or in a protein with multiple functions. The interallelic complementation observed for these three cases are not readily explained if the only essential role of Cdc4p is as an ECL, because the ECL acts monomerically. Other evidence that Cdc4p may have multiple functions is that only the *cdc4*^{R33K} allele is impaired for actin filament gliding by Myo2p, whereas the other *cdc4* ts alleles are not impaired for this function (Lord and Pollard, 2004). It thus seems that while *cdc4* is involved in cytokinesis *via* acting as an ELC it is also involved in other roles. In addition to the genetic complexity, the structural study suggests that the Cdc4 N-terminal and C-terminal domains may differentially interact with partner protein(s) (Escobar-Cabrera *et al.*, 2005). In fact, Cdc4p has been reported to physically interact with numbers of proteins (Desautels *et al.*, 2001; D'souza *et al.*, 2001). Among Cdc4p-interacting partners, a putative type IIIβ PtdIns 4-kinase Pik1p was identified as a Cdc4p-binding protein. The C-terminal 345 amino acids of Pik1p were recovered in a yeast two-hybrid screen (Desautels *et al.*, 2001). This interaction has not been reported in other organisms.

As described in section 1.3.3.2., the functions of orthologues of *S. pombe* Pik1p in other organisms have been studied. In particular, the orthologues in *S. cerevisiae* and *Drosophila* are involved in cytokinesis but the specific aspects of cytokinesis that are regulated by these proteins are not certain. I thus wished to determine the biological functions of Pik1p in *S. pombe*. I hypothesize that *S. pombe* Pik1p functions in cytokinesis because of the observation that *S. pombe* Cdc4p interacts with Pik1p and the

observations made in other organisms. This is the first study of the biological functions of the *S. pombe* type IIIβ PtdIns 4-kinase, Pik1p.

1.4.2. Specific objectives:

1.4.2.1. Are *S. pombe pik1* functions conserved?

Comparisons of aligned amino acid sequences of type IIIβ PtdIns 4-kinase C-terminal catalytic domains showed that they are conserved among organisms such as yeasts, insects, slime mold, animals and plants (Gehrmann and Heilmeyer, Jr., 1998; Brill *et al.*, 2000; Desautels *et al.*, 2001). I wished to determine if the essential functions of *S. pombe* Pik1p are conserved. To answer this question, a complementation study was performed. An expression vector carrying an *S. pombe pik1* cDNA sequence was introduced into *S. cerevisiae* cells carrying a conditional ts allele, *pik1-101*. Colony formation was performed to assess whether the heterologous *S. pombe pik1* provided the lost essential functions of the *S. cerevisiae pik1-101* ts mutant at the restrictive temperature.

1.4.2.2. Is *S. pombe pik1* essential for cell viability?

The *S. pombe* and *S. cerevisiae* genomes each appear to include three PtdIns 4-kinases paralogues (Wood *et al.*, 2002; de *et al.*, 2005). *PIK1*, the *S. cerevisiae* orthologue of *S. pombe pik1*, is essential for cell viability (Flanagan *et al.*, 1993). The orthologue in *Drosophila*, *fwd*, is not essential (Brill *et al.*, 2000). It was not known if *S. pombe pik1* is essential for cell viability. To determine this, a gene deletion study in diploid cells and tetrad dissection analysis was conducted. Also, plasmid-loss in haploid cells carrying a deletion of the *pik1* chromosomal coding region and an episomal *pik1* sequence was performed.

1.4.2.3. Is *S. pombe pik1* involved in cytokinesis?

As described above, some phosphoinositides, lipid kinases and lipid phosphatases are involved in cytokinesis. In addition, our finding that the essential actomyosin ring component Cdc4p interacts with the *S. pombe* Pik1 C-terminal domain suggests that *S. pombe* Pik1p may directly or indirectly participate in cytokinesis. The

availability of a conditional loss-of-function *pik1* allele would aid the investigation of this question. In the case where *S. pombe pik1* proves to be essential, a conditional loss-of-function *pik1* allele might reveal terminal phenotypes under the restrictive condition, which would indicate potential Pik1p physiological functions.

1.4.2.4. Is the localization of Pik1p in *S. pombe* consistent with a role in cytokinesis?

The localization of effecters often suggests their roles in specific cellular processes. In particular, examination of protein distribution during the cell division cycle can be informative. Along these lines, the localization of Pik1p in *S. pombe* cells was determined. A common approach is fluorescence microscopy of live or fixed cells that contain a tagged protein.

1.4.2.5. Does *S. pombe* Pik1p interact with other protein(s) involved in cytokinesis?

Type IIIβ PtdIns 4-kinases have been reported to interact with several protein partners (Hendricks *et al.*, 1999; Haynes *et al.*, 2005). *S. pombe* Pik1p may also interact with other protein(s) to function in cellular processes, especially during cytokinesis. For example, the Pik1 C-terminal domain interacts with Cdc4p in a yeast two-hybrid screen. Targeting proteins to specific locations and orchestrating numerous protein-protein interactions are required for the temporal and spatial control of cytokinesis. Therefore, the use of a tandem-affinity-purification (TAP) tagging method was investigated for the identification of proteins that interact with Pik1p.

Chapter 2: Materials and Methods

2.1. Yeast

2.1.1. *S. pombe* and *S. cerevisiae* strains used in this project

S. pombe and *S. cerevisiae* strains used in this study are listed in Table 2.1. and Table 2.2., respectively. The *S. pombe* haploid strains, N2 and N3, were used to generate diploid cells. The diploid cells were used for deletion of the genomic *pik1* coding region by homologous recombination (see section 2.1.10.1.) to generate the hemizygous diploid strain N1231. The latter strain was transformed with various episomes and sporulated to generate haploid cells with a deleted chromosomal *pik1* coding locus but expressing various episomal *pik1* alleles including tagged alleles.

Strains N1255 and N1285 (*S. cerevisiae PIK1* temperature-sensitive mutant and wild-type, respectively) were generously provided by P. Novick (Yale University, New Haven, USA) and W. Xiao (University of Saskatchewan, Saskatoon, Canada), respectively.

Table 2.1. *S. pombe* strains

Strain	Genotype	Source
N2	h^+ *ade6-M210 leu1-32 ura4-D18*	Lab Stock
N3	h^- *ade6-M216 leu1-32 ura4-D18*	Lab Stock
N1095	pREP81-*pik1* in N2	Lab Stock
N1113	h^+ Δ*pik1*::Kan^R *ade6-M210 leu1-32 ura4-D18*, pREP81-*pik1*	This study
N1231	h^+/h^- *pik1*/Δ*pik1*::*ura4 ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study
N1240	h^- Δ*pik1*::*ura4 ade6-M210 leu1-32 ura4-D18*, pREP41-NTAP-*pik1*	This study
N1361	pREP41-NTAP in N3	This study
N1364	pREP41X-N degron-*pik1* in N1231	This study
N1366	h^- Δ*pik1*::*ura4 ade 6-M216 leu1-32 ura4-D18*, pREP41X-N degron-*pik1*	This study
N1369	h^+ Δ*pik1*::*ura4 ade6-M210 leu1-32 ura4-D18*, pREP41-2XeGFP-*pik1*	This study
N1373	h^- Δ*pik1*::*ura4 ade6-M216 leu1-32 ura4-D18*, pREP41-*pik1*	This study
N1401	h^- *cdc25-22 leu1-32*, pREP41-2XeGFP-*pik1*	This study

Table 2.2. *S. cerevisiae* strains

Strain	Genotype	Source
N1255	*MAT*a *ura3-52 leu2-3,112 GAL*$^{+}$ *pik1-101*	P. Novick
N1285	*MAT*a *trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112 ade2-1*	W. Xiao
N1301	YEplac181+ P_{nmt1}-*pik1*-T_{nmt1} in N1285	This study
N1302	YEplac181+ P_{nmt1}-*pik1*-T_{nmt1} in N1255	This study
N1310	YEplac181 in N1285	This study
N1311	YEplac181 in N1255	This study
N1312	YEplac181 + P_{nmt41}-eGFPN-*pik1* cDNA-T_{nmt} in N1285	This study
N1313	YEplac181 + P_{nmt41}-eGFPN-*pik1* cDNA-T_{nmt} in N1255	This study
N1314	YEplac181+ P_{nmt81}-*pik1* cDNA-T_{nmt1} in N1285	This study
N1315	YEplac181+ P_{nmt81}-*pik1* cDNA-T_{nmt1} in N1255	This study
N1322	YEplac181 + P_{nmt41}-*pik1* cDNA-T_{nmt1} in N1285	This study
N1323	YEplac181 + P_{nmt41}-*pik1* cDNA-T_{nmt1} in N1255	This study
N1343	YEplac181-P_{nmt41}-*pik1*^{D709A}-T_{nmt1} in N1285	This study
N1344	YEplac181-P_{nmt41}-*pik1*^{D709A}-T_{nmt1} in N1255	This study
N1362	YEplac181-P_{nmt41}-eGFP-*pik1*^{D709A}-T_{nmt1} in N1255	This study
N1363	YEplac181-P_{nmt41}-eGFP-*pik1*^{D709A}-T_{nmt1} in N1285	This study
N1467	YEplac181-P_{nmt41}-eGFP-*pik1*^{R838A}-T_{nmt1} in N1285	This study
N1468	YEplac181-P_{nmt41}-eGFP-*pik1*^{R838A}-T_{nmt1} in N1255	This study

Table 2.3. Reagents

Media	
YES	BIO 101 #4101-532, CA, USA
EMM	BIO 101 #4110-032, CA, USA
ME	BIO 101 #4103-012, CA, USA

Reagents	
amino acids (adenine, lysine, leucine, histidine, uracil)	Sigma, St. Louis, USA
Thiamine	Sigma T-1270, St. Louis, USA
Glucose	Sigma G-8270, St. Louis, USA
Litium acetate	Sigma L-4158, St. Louis, USA
paraformaldehyde	Sigma P-6148, St. Louis, USA
Bacto-peptone	DIFCO 0120-01, MD, USA
Bacto-Tryptone	DIFCO 211705, MD, USA
Bacto-Yeast extract	DIFCO 212750, MD, USA
G418 (Geneticin)	GIBCO 11811-031, Burlington, Canada
SDS	GIBCO 15525-017, Burlington, Canada
Sorbitol	Fisher Scientific S495-500, ON, Canada
MgCl2	Fisher Scientific M33B, ON, Canada
NaOH	Fisher Scientific S318B, ON, Canada
EDTA	EMD EX0539-1, Germany
NaCl	EMD SX0420-3, Germany
Potassium acetate	EMD PX1330-1, Germany
Tris-Base	Roche 03 118 142 001, IN, USA
Glycerol	Anachemia 43567-360, QC, Canada

Restriction enzymes	
*Nde*I	New England BioLabs R0111L, MA, USA
*Xba*I	New England BioLabs R0145S, MA, USA
*Xho*I	New England BioLabs R0146S, MA, USA
*Bam*HI	Invitrogen 15201-031, Burlington, Canada
*Pst*I	Invitrogen 15215-023, CA, USA
*Sst*I	Invitrogen 15222-011, Burlington, Canada
*Bgl*II	Invitrogen 15213-028, Burlington, Canada
*Sma*I	Invitrogen 15228-018, Burlington, Canada
*Kpn*I	GIBCO 15232-036, Burlington, Canada

2.1.2. *S. pombe* haploid cell culture, media and culture conditions, asynchronous cultures

Growth media (YES, and EMM with supplements [225 mg/L of each of adenine, lysine, leucine, histidine and uracil, as appropriate]) were as described in Moreno *et al*. (1991). *S. pombe* haploid cells were cultured in rich medium, YES, or minimal medium, EMM with histidine, lysine, adenine, uracil but lacking leucine (for cells with *LEU2* selectable marker), or EMM with histidine, lysine, adenine, leucine but lacking uracil (for cells with *ura4* selectable marker) according to their genotypes at 30˚C. Cells with temperature-sensitive alleles were cultured at a permissive temperature of 25˚C or a restrictive temperature of 36˚C. For precultures, cells from a single colony were incubated in 10 mL medium overnight with shaking (300 rpm). The cultures were started from the precultures at cell density 1 x 10^5 cells/mL.

2.1.3. *S. pombe* diploid cell culture, media and culture conditions, asynchronous cultures

Growth media (ME, SPA, and EMM with supplements) were as described (Moreno *et al.*, 1991; Leupold, 1970). Incubation of diploid cells on ME or SPA plates was used to induce meiosis and sporulation (see section 2.1.3.). The appropriate media used for diploid cells are described in the legend under the each figure. Briefly, *S. pombe* diploid cells were cultured in EMM media lacking adenine; adenine and uracil; or adenine, uracil, and leucine according to their genotypes at 30˚C. Cells were incubated in minimal medium EMM lacking adenine. Haploid cells with *ade*6-M210 or *ade*6-M216 alleles do not grow under these conditions while diploid cells do by virtue of interallelic complementation (Szankasi *et al.*, 1988).

2.1.4. *S. pombe cdc25-22* haploid cell culture, block-and-release synchronization

S. pombe cdc25-22 cells were used for temperature block-and-release synchronization. The *cdc*25 gene encodes a protein phosphatase, which is required to activate MPF at the onset of mitosis. Typically, cells were incubated at the permissive temperature (25˚C) for 18-22 hours. Cells were then incubated for 4 hours at 36˚C, which blocked progression through the cell cycle at G2/M, and collected by

centrifugation (5 minutes at 3000 x g). Medium (50 mL), pre-warmed to 25˚C, was added and the cultures were incubated for 3 hours with aliquots taken every 20-30 minutes. Release of cells from 36˚C to 25˚C allowed cells to synchronously enter M phase (Nurse *et al.*, 1976).

2.1.5. *S. pombe* cell mating and random spore analysis

Cell mating and random spore analysis were used to generate diploid cells and haploid cells possessing desirable genotypes for experimental purposes. Haploid cells with the opposite mating type (h^+ or h^-) were streaked onto YES. Distilled water (25 μL) was placed on SPA or ME media plate. Cells from a single colony each of haploid h^+ and h^- cells were mixed with the drop of water, which was allowed to evaporate. The culture plate was incubated at 25˚C. To obtain diploid cells, the culture was kept for 2 days at 25˚C. Cells from the mixture were streaked on an EMM plate lacking adenine and incubated at 30˚C until the appearance of colonies (5 days). A colony was then re-streaked on a fresh EMM –adenine plate before use and / or storage.

For random spore analysis, 2 x 10^7 diploid cells per 25 μL of sterile distilled water were incubated on SPA or ME medium at 25˚C for 4 - 5 days. Cells and asci were collected and resuspended in 1 mL of sterile distilled water. A 5 μL of β-glucuronidase from *Helix pomatia* (Sigma G-7770, St. Louis, USA) was added. The mixture was incubated overnight at a room temperature with agitation. The β-glucuronidase is used to break the ascus wall and kill vegetative cells in order to isolate spores. The spores were washed three times with sterile distilled water. The spores were diluted 1/500, 1/1000, 1/2000, and 1/5000 fold and 50 μL aliquots of each dilution were spread on minimal media EMM plates lacking supplements according to genotype. The plates were incubated at 30˚C (25°C for ts mutants) until colonies formed. One colony was re-streaked onto a fresh media plate before use and storage.

2.1.6. *S. pombe* transformation, lithium acetate method

Cell transformation by the lithium acetate method was as described by Moreno *et al*. (Moreno *et al.*, 1991). Briefly, cells grown in EMM with appropriate supplements to a density of 0.5 – 1 x 10^7 cells/mL were harvested at 3000 x g for 5 minutes. The cells

were washed with 40 mL sterile distilled water, and collected by centrifugation. The cells were resuspended at 1 x 10^9 cells/mL in 0.1 M lithium acetate, which was adjusted to pH 4.9 with acetic acid, and were dispensed as 100 µL aliquots into Eppendorf tubes. These aliquots were incubated at 30°C (25°C for ts mutants) for 1 – 2 hours. 1 µg of plasmid DNA in 15 µL TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) was added. After gentle mixing by finger tapping, 290 µL of 50% (w/v) polyethylene glycol (PEG) 4000 prewarmed to 30°C (25°C for ts mutants) was added and the cells were incubated at 30°C (25°C for ts mutants) for 1 hour. This was followed by heat shock at 43°C for 15 minutes, followed by a cool down period at room temperature for 10 minutes. The cells were collected by centrifugation and resuspended in 1 mL of half strength YE (1/2 YE) medium by gentle pipetting up and down. 1/2 YE medium (9.0 mL) and the cells were incubated with shaking at 30°C (25°C for ts mutants) for at least 1 hour. Cells were plated on EMM lacking adenine and leucine; or adenine, uracil, and leucine, as applicable. A colony was restreaked on a fresh plate prior to use and storage.

2.1.7. *S. pombe* transformation, electroporation

Fission yeast transformation by electroporation was as described by Suga and Hatakeyama (2001). Briefly, cells were cultured to the mid-logarithmic phase of growth in EMM with appropriate supplements at 30°C (25°C for ts mutants), recollected by centrifugation, and washed with sterile ice-cold distilled water. A second wash was performed with sterile ice-cold 1 M sorbitol solution. After centrifugation to collect cells (3000 x g for 5 minutes), cells were resuspended in DTT buffer (25 mM DTT, 0.6 M sorbitol, 20 mM Hepes pH7.5), and incubated at 30°C for 15 minutes. These cells were washed 3 times with ice-cold 1 M sorbitol solution. Cells (40 µL) at a density of 1-5 x 10^9 cells/mL and plasmids (about 1 µg) were mixed into a pre-chilled cuvette (disposable 2 mm Gap Cuvette, BTX, San Diego, USA), and incubated on ice for 5 minutes. The electroporator (BioRad Gene Pulser™ 165-2076, USA) settings were: 200 ohms resistance, 25 µFD Capacitance, and 2.5 kV volts. Immediately after the pulse, 300 µL of ice-cold 1 M sorbitol was added, and the cells were transferred into a culture flask with 10 mL of EMM with appropriate supplements. The cells were incubated at 30°C (25°C for ts mutants) for about 24 hours. These cells were plated on EMM with

appropriate supplements, and incubated at 30°C (25°C for ts mutants). A colony was restreaked on a fresh plate prior to use and storage.

2.1.8. *S. cerevisiae* transformation

A lithium acetate transformation method was described in Gietz and Woods (2002). *S. cerevisiae* cells were cultured overnight in 10 mL of YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose; W/V) medium. Single-stranded carrier DNA (2 mg/mL herring sperm DNA) was prepared by boiling for 5 minutes followed by immediately chilling on ice. The transformation mix [T Mix; 240 μL of 50% (W/V) PEG 3350, 35 μL of 1.0 M lithium acetate pH 4.9, 50 μL of 2 mg/mL single-stranded carrier DNA, 34 μL of plasmid DNA (about 1 μg) in distilled water] was added to spun-down cells and adjusted to a final volume 360 μL with distilled water. The cells were gently mixed by pippetting up and down, and incubated at 42°C for 1 hour. Cells were collected by centrifugation at 13000 x g at room temperature for 1 minute. The cells were resuspended into 500 – 1000 μL of distilled water, and plated on minimal media (SD lacking supplements). The plates were incubated at 30°C (25°C for ts mutants) until the colonies formed. A colony was restreaked on a fresh plate prior to use and storage.

2.1.9. Complementation analysis

Complementation analysis was performed to assess whether the essential function(s) of *S. pombe* Pik1p and *S. cerevisiae* Pik1p is(are) conserved. In addition, the importance of two *S. pombe pik1* residues (D709 and R838) to provide the essential function(s) of Pik1p was evaluated. The wild-type *pik1* coding sequence and *pik1* allelic mutant (*pik1*^{D709A} and *pik1*^{R838A}) sequences were introduced into a conditional temperature-sensitive *S. cerevisiae pik1-101* mutant or a wild-type *S. cerevisiae* haploid. I used the *S. cerevisiae pik1-101* strain because it is well characterized (Walch-Solimena and Novick, 1999). This allele carries a single mutation that produces the substitution $1045^{Ser \rightarrow Phe}$.

2.1.9.1. Construction of plasmids

A cloned *S. pombe pik1* cDNA was provided by S. Steinbach. An *Nde*I site internal to the coding region was removed by introduction of a silent T to C mutation at nucleotide 300. Restriction sites for *Nde*I and *Bam*HI were introduced at the 5′ and 3′ ends of the coding region, respectively. This cDNA with the silent mutation is referred as a 'wild-type' sequence (*pik1*) of the *pik1* cDNA in this dissertation. The coding regions from *S. pombe* wild-type *pik1*, or *pik1*^{D709A} or *pik1*^{R838A} cDNA sequences were cloned between the *Nde*I and *Bam*HI sites of *S. pombe* expression vectors; pREP1, pREP41 and pREP81 (Maundrell, 1993). A cDNA encoding a wild-type amino acid sequence was also cloned between the *Nde*I and *Bam*HI sites of pREP41-eGFP N (Craven *et al.*, 1998). A thiamine-repressible *nmt1* promoter sequence directs expression from the pREP1 plasmid. The promoter strength is attenuated in the pREP41 vectors and strongly attenuated in pREP81 (Forsburg, 1993; Maundrell, 1993). The *S. pombe pik1* coding regions flanked by *nmt1* promoter and terminator sequences were cloned between the *Pst*I and *Sst*I sites of the *S. cerevisiae* expression vector, YEplac181 (Gietz and Sugino, 1988) (Figure 2.1. A, page 63). The eGFP fused *pik1* coding region flanked by the attenuated *nmt1* promoter and terminator sequences was cloned between the *Pst*I and *Sst*I sites of the YEplac181 (Figure 2.1. A).

I constructed a plasmid YEplac181-P_{nmt41}-eGFP-*pik1*^{D709A}-T_{nmt}. When the *pik1* D709 residue was originally mutated to alanine using site-directed mutagenesis, an internal *Pst*I site was introduced into the *pik1* cDNA for the selection of the mutated allele (Steinbach *et al*., unpublished). The following cloning procedures were used: step 1, an internal *Pst*I – *Sst*I fragment from pREP41-*pik1*^{D709A} was cloned into the YEplac181; step 2, a *Pst*I – internal *Pst*I fragment from pREP41-*pik1*^{D709A} was cloned into the plasmid product of the first step; step 3, the orientation of a *Pst*I – internal *Pst*I fragment was confirmed by restriction enzyme digestion; step 4, a *Nde*I – *Nde*I fragment of a PCR-amplified eGFP coding sequence was cloned into the plasmid product of the third step; step 5, the orientation of a *Nde*I – *Nde*I fragment was confirmed by restriction enzyme digestion. The final construct is drawn in Figure 2.1. B.

To obtain plasmid YEplac181-P_{nmt41}-eGFP-*pik1*^{R838A}-T_{nmt}, a *Bgl*II – *Sst*I fragment with the R838A residue was obtained from a plasmid pREP41-*pik1*^{R838A}. A plasmid YEplac181-P_{nmt41}-eGFP-*pik1*^{D709A}-T_{nmt} was digested by using *Bgl*II and *Sst*I to

remove a fragment with the D709A residue (YEplac181- P_{nmt41}-eGFP-*pik1*). The *Bgl*II – *Sst*I fragment with the R838A residue was cloned between the *Bgl*II and *Sst*I sites of YEplac181-P_{nmt41}-eGFP-*pik1*. The description of this plasmid is shown in Figure 2.1. B. The plasmids are listed in Table 2.4.

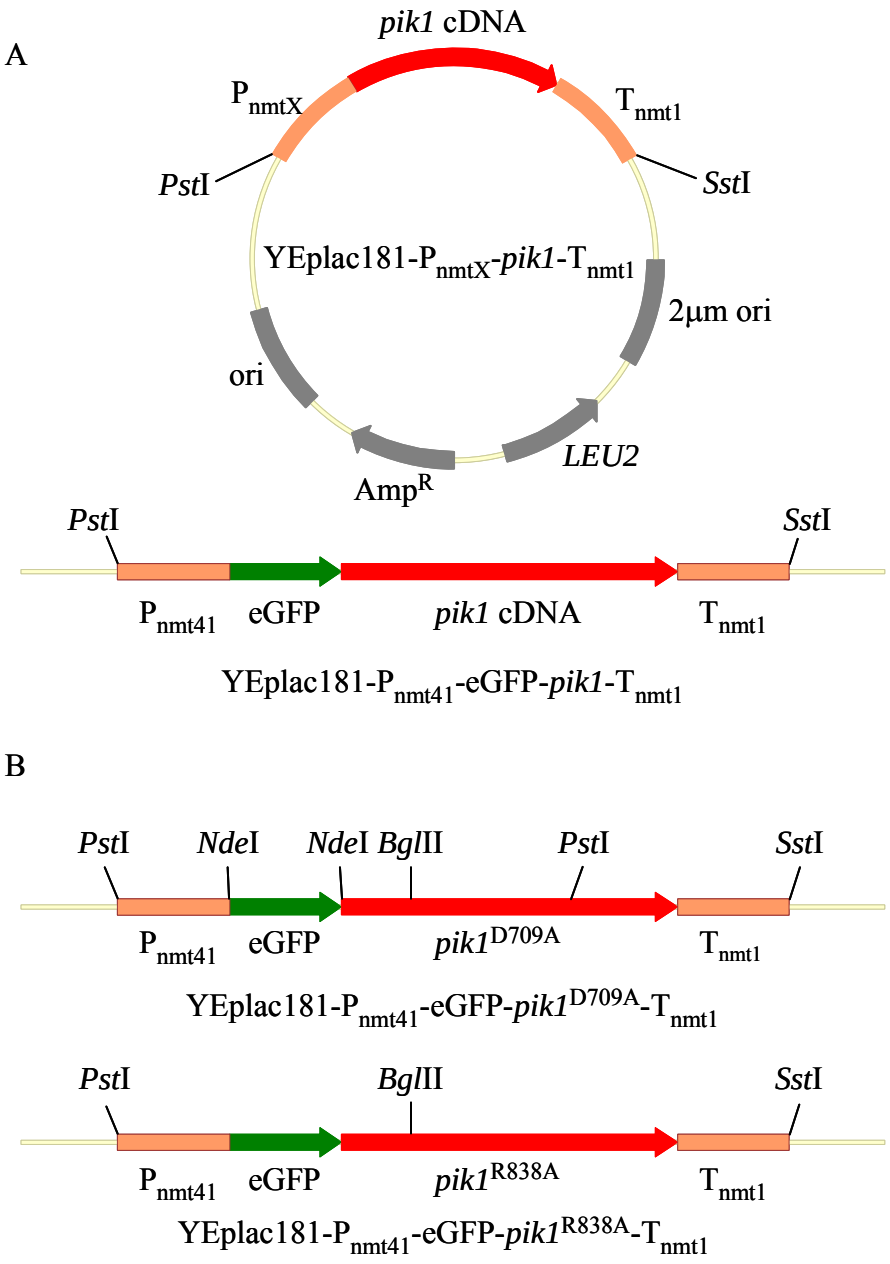


Figure 2.1. Plasmids for complementation studies in *S. cerevisiae*. The plasmid, YEplac181, used for these studies is an *S. cerevisiae* – *E. coli* shuttle vector. (A) Restriction enzyme sites that were used for cloning are designated. Abbreviations, Amp^R and *LEU2*, stand for an ampicillin-resistance gene expression cassette and *S. cerevisiae LEU2* gene expression cassette, respectively. For plasmid YEplac181-P_{nmt41}-eGFP-*pik1*-T_{nmt}, only the insert is different. P_{nmtx} is the *S. pombe nmt*1 promoter or two attenuated versions. X stands for 1, 41, or 81. T_{nmt1} is the *S. pombe nmt*1 terminator. *S. pombe pik1* cDNA was obtained by Steinbach. This cDNA possesses a silent mutation, $His^{300T\rightarrow300C}$ in order to eliminate an internal *Nde*I restriction site. (B) For plasmids YEplac181-P_{nmt41}-eGFP-$pik1^{D709A}$-T_{nmt1} and YEplac181-P_{nmt41}-eGFP-$pik1^{R838A}$-T_{nmt1}, only the inserts are different.

Table 2.4. Plasmids

Plasmid	Source
pFA6a-kanMX6	Lab stock
KS-*ura4*	Lab stock
pREP1-*pik1*	Lab stock
pREP41-*pik1*	Lab stock
pREP81-*pik1*	Lab stock
pBluescript KS(-) - 656nt+*Kan*^{R}+610nt	This study
pBluescript KS(-) - 656nt+*ura4*+610nt	This study
YEplac181	W. Xiao
YEplac181-P_{nmt1}-*pik1*-T_{nmt}	This study
YEplac181-P_{nmt41}-*pik1*-T_{nmt}	This study
YEplac181-P_{nmt81}-*pik1*-T_{nmt}	This study
YEplac181-P_{nmt41}-eGFP-*pik1*-T_{nmt}	This study
YEplac181-P_{nmt41}-eGFP-*pik1*^{D709S}-T_{nmt}	This study
YEplac181-P_{nmt41}-eGFP-*pik1*^{R838A}-T_{nmt}	This study
pREP41-2XeGFPN-*pik1*	This study
pREP41-Ub-R-DHFR^{ts}-*pik1*	This study
pREP41-NTAP	K. Gould
pREP41-NTAP-*pik1*	This study

2.1.9.2. *S. cerevisiae* cell culture and serial dilution

S. cerevisiae wild-type (strain N1285) and mutant (strain N1255) cells were transformed with the plasmids shown (Figure 2.1., page 63) using a lithium acetate transformation method (described in section 2.1.4.2.). All *S. cerevisiae* transformants (Table 2.2., page 55) were cultured in synthetic minimal medium lacking leucine (SD-leucine; 0.67% nitrogen base, 2% glucose, and amino acid supplements as appropriate) in the presence or absence of 15 μM thiamine (Table 2.3., page 56). The thiamine was used because it suppresses expression from the *S. pombe nmt1* promoter. For colony formation assays, cells were precultured in 1.5 mL medium with thiamine at 25°C overnight. Cells were counted and cultures started at an initial cell density of 1 x 10^5 cells/mL in 50 mL at 25°C. After 18-20 hours, cells were recovered by centrifugation (13000 x g for 1 minute), washed 3-times in sterile distilled water, and resuspended at a cell density of 2 x 10^7 cells/mL. The cells were serially diluted 10-fold and 5 μL of each dilution were spotted onto SD-leucine with or without thiamine plates and incubated at a permissive (25°C) and restrictive (37°C) temperature for 5 days.

2.1.10. *pik1* gene deletion in *S. pombe* cells

To evaluate whether *S. pombe pik1* is an essential gene, two gene deletion studies were performed: (1) replacement of the entire *pik1* genomic coding region from one chromosome in diploid cells followed by sporulation and tetrad analysis and (2) replacement of the entire *pik1* genomic coding region in haploid cells containing an episome pREP81-*pik1* for conditional expression of the *pik1* gene.

2.1.10.1. Deletion of genomic *pik1* by homologous recombination

The 656 bp DNA sequence immediately upstream of the *pik1* coding region (SPAC22E12.16c) was PCR amplified with incorporation of restriction sites, *Xba*I /*Bam*HI, with the primer set H1393/H1399, resulting in *Xba*I – 656bp – *Bam*HI fragments. The 610 bp of DNA sequence immediately downstream of the *pik1* coding region (SPAC22E12.16c) was PCR amplified with incorporation of restriction sites, *Bam*HI/*Kpn*I, with the primer set H1400/H1401, resulting in *Bam*HI – 610pb – *Kpn*I fragments. A *ura4* expression cassette DNA sequence was PCR amplified with

incorporation of restriction sites, *Bam*HI/*Bam*H I using the primer set H1371/H1404, resulting in *Bam*HI – *ura4* – *Bam*HI fragments. The three DNA fragments (the 656 bp sequence 5´ of the *pik1* coding region, the *ura4* expression cassette, and the 610 bp sequence 3´ of the *pik1* coding region) were cloned into pBluescript KS- (Stratagene 212208, La Jolla, USA) (pBluescript KS(-) – 656 bp-*ura4*-610 bp, Figure 2.2. A, page 81). Alternatively, a kanamycin-resistance gene expression cassette (Kan^R) DNA sequence was PCR amplified with incorporation of restriction sites, *Bam*HI/*Bam*HI (a primer set H1402/H1403), instead of a *ura4* expression cassette (Figure 2.2. A). The PCR amplification conditions are described in section 2.2.4. The primer sequences are listed in Table 2.5 (page 76). The PCR products were purified using a commercial purification kit (Qiagen 28106, Mississauga, Canada). Alternatively the PCR products were separated from the reactants by agarose gel electrophoresis and isolated with a commercial gel purification kit (Qiagen 28706, Mississauga, Canada).

Homologous recombination was then used to replace the entire coding region of *pik1* by the *ura4* cassette in diploid cells or by the Kan^R cassette in haploid cells containing an episome pREP81-*pik1* (Figure 2.2. B). A linear *Xba*I – *Kpn*I fragment (10 µg) was used for cell transformation as described in section 2.1.4.1. Diploid cells (h^+/h^- *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*) and haploid cells (strain N1095) were transformed with the *Xba*I – *Kpn*I fragments. Transformed diploid cells were plated on EMM lacking adenine and uracil. Transformed haploid cells were plated on YES containing 100 mg/L of G418 (aminoglycoside antibiotic geneticin) (Table 2.3., page 56). The plates were incubated at 30°C until colonies formed. Single colonies were restreaked on fresh plates prior to use and storage.

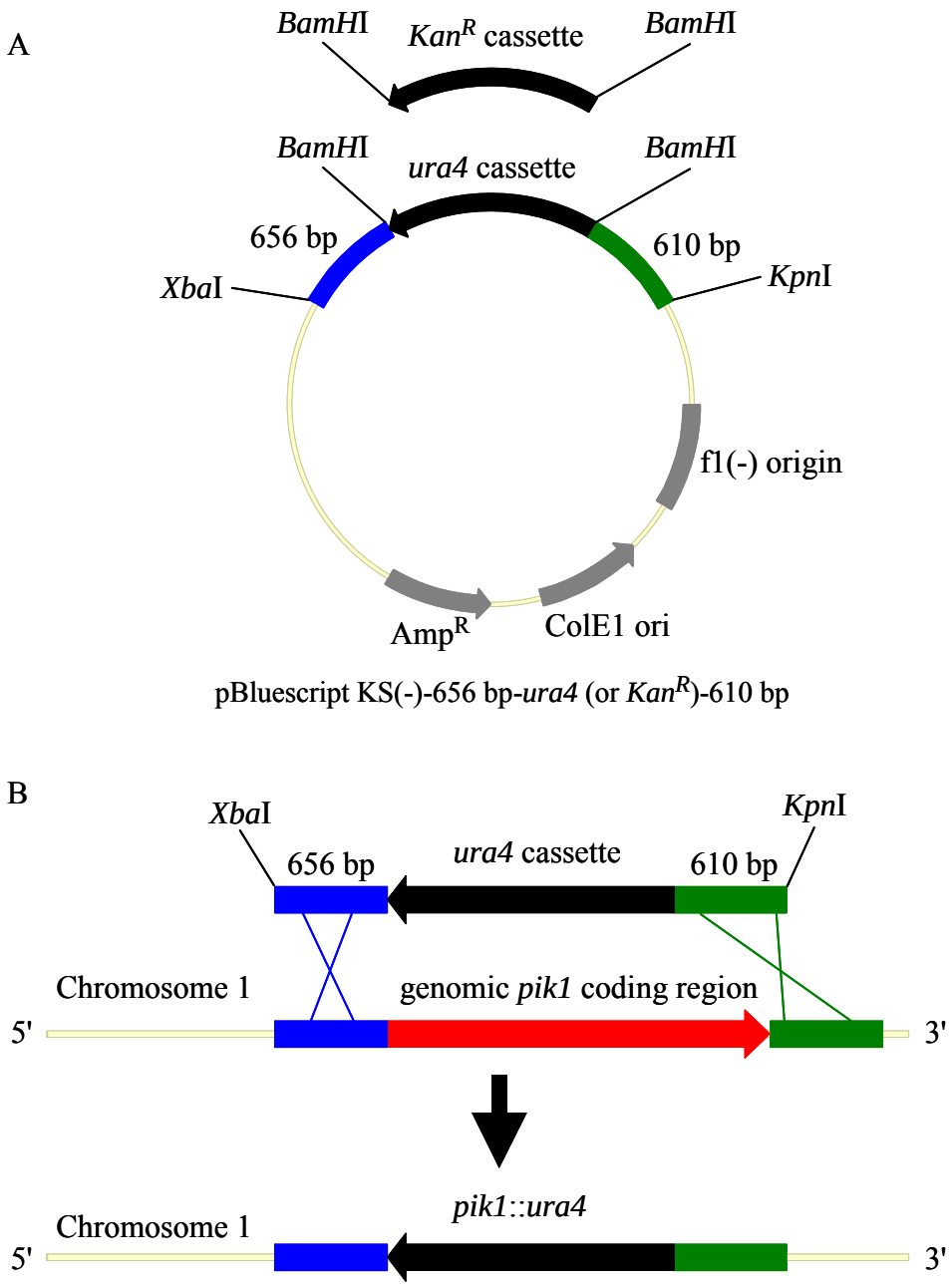


Figure 2.2. Genomic *pik1* locus deletion studies in *S. pombe*. (A) Constructs of plasmids. Two *pik1* gene deletion plasmids differ only in the selectable marker employed. These two selectable markers were inserted in the opposite orientation to the genomic *pik1* locus. Restriction enzyme sites used for cloning are designated. "656 bp" refers to sequences upstream of the *pik1* genomic coding region. "610 bp" refers to sequences downstream of the *pik1* genomic coding region. ColE1 ori is an *E. coli* replication origin. (B) As a result of homologous recombination, the coding region of the genomic *pik1* locus in chromosome 1 was replaced with the *ura4* cassette or Kan^R cassette.

2.1.10.2. Tetrad dissection

The tetrad dissection method was described by Moreno *et al.* (Moreno *et al.*, 1991). Diploid cells were incubated on SPA (Leupold, 1970) or ME plates at 25°C for 2 days, resulting in ascus production. These asci were restreaked on one side of a thin-layered YES plate, and incubated at 36°C for about 4 hours. Using a ZEISS Tetrad Micromanipulator, 8-10 asci were isolated and placed in a line. The asci walls were ruptured by repeated manipulations and the four spores from each ascus were separated from each other. The YES plates were incubated at different temperatures (19°C, 25°C, 30°C, and 36°C) until the appearance of colonies.

2.1.11. Conditional temperature-sensitive *S. pombe pik1* strains

As *pik1* proved to be an essential gene (section 3.2.), it was important to generate a conditional temperature-sensitive *pik1* mutant to examine what aspects of cell growth and division are primarily affected by the loss of *pik1* function. Two approaches were used: (1) site-directed mutagenesis of the *S. pombe pik1* gene and (2) fusion of a thermolabile tag to *pik1*.

2.1.11.1. Generation of a *pik1* ts mutant by site-directed mutagenesis

A single residue replacement ($1045^{Ser \rightarrow Phe}$) confers conditional temperature-sensitive characteristics in the *S. cerevisiae pik1-101* mutant (Walch-Solimena and Novick, 1999). The C-terminal sequences of *S. pombe* Pik1p and *S. cerevisiae* Pik1p are highly conserved (Desautels *et al.*, 2001). I therefore wished to replace the corresponding residue in *S. pombe* Pik1p ($Pik1^{A831F}$; Figure 3.18., page 130).

Site-directed mutagenesis was performed as described (Kunkel *et al.*, 1991). A single-stranded *pik1* coding sequence containing uracil residues in plasmid pRSET-B was available in the laboratory (S. Steinbach). To introduce the R831F mutation into *pik1* cDNA, oligonucleotide H1616 was used. The H1616 oligonucleotide is 60-nt long and possesses a codon replacement encoding Ala to Phe at residue 831. Oligonucleotide H1616 was first phosphorylated for subsequent ligation, and then hybridized to the uracil-containing single-stranded DNA template (pRSET-B-*pik1*), which was followed by *in vitro* DNA synthesis. Bacterial transformation was performed with the products, *in*

vitro synthesized double-stranded pRSET-B-*pik1*. The uracil-containing DNA template strand was degraded leaving the newly synthesized strand for plasmid amplification. A plasmid extraction was done as described in section 2.2.5.

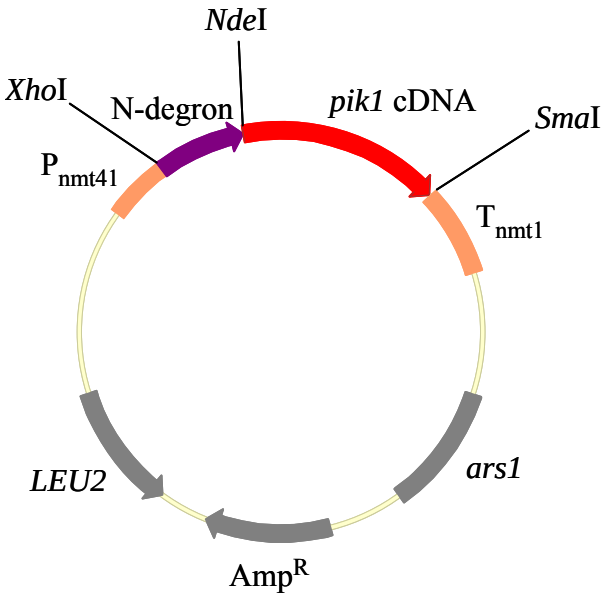
2.1.11.2. Thermolabile *S. pombe pik1-td* strain

This approach is based on conditional proteolysis of a protein of interest, whose N-terminus is fused to an N-degron tag. The N-degron tag is made of a ubiquitin sequence, arginine, and a temperature-sensitive mouse dihydrofolate reductase (Ub-R-DHFR^{ts}; N-degron, (Dohmen *et al.*, 1994). The DHFR^{ts} conformation is altered upon temperature shift to 36°C, leading to the exposure of its internal lysines toward cytoplasm. The exposed lysines are polyubiquitinated for ubiquitin-dependent proteolysis at 36°C. To be of use, the N-degron fused protein is supposed to be functional at 25°C, but at 36°C it should be removed by proteolysis (Figure 3. 19., page 132). I therefore generated haploid cells lacking a chromosomal *pik1* locus but containing an episomal N-degron fused *pik1* allele in order to confer a conditional temperature-sensitive *pik1* allele (section 3.3.2.1.).

2.1.11.2.1. Construction of plasmid, pREP41X-Ub-R-DHFR^{ts}-*pik1*

The plasmid pCDL674, originally constructed by Dohmen *et al.* (*S. cerevisiae* plasmid pPW66R) (Dohmen *et al.*, 1994), was generously provided by M. Balasubramanian. This plasmid contains an N-degron tag (Ub-R-DHFR^{ts}). The plasmid pCDL674 was used as the template DNA to obtain the N-degron DNA sequences. About 800 bp of the N-degron DNA sequence were PCR amplified with incorporation of restriction sites, *Xho*I and *Bgl*II using the primer set H1608/H1609. To generate the restriction sites on the 5′ and 3′-ends of the *pik1* cDNA, PCR amplification was performed with the template pREP1-*pik1* sequence and the primer set H1603/H1607, giving a *Bam*HI site on the 5′-end and a *Sma*I site on the 3′-end. The cloning process was as follows: step 1, the *pik1* cDNA fragment was first inserted between *Bam*HI and *Sma*I sites of pREP41X (Craven *et al.*, 1998); step 2, the *Xho*I-*Bam*HI N-degron fragment was ligated between *Xho*I and *Bam*HI sites of the plasmid pREP41X-*pik1* so

that the overhangs of *Bam*HI and *Bgl*II sites for ligation are complementary. The constructed plasmid is shown in Figure 2.3.



pREP41X-Ub-R-DHFR^{ts}-*pik1*

Figure 2.3. Schematic representation of pREP41X-Ub-R-DHFR^{ts}-*pik1* expression plasmid for the *pik1-td* allele. The N-degron tag is composed of sequences fused in-frame that encode ubiquitin (Ub), Arg (R), and temperature-sensitive dihydrofolate reductase (DHFR^{ts}). Restriction enzyme sites used for cloning are designated. Plasmid pREP41X possesses an *Xho*I site instead of *Nde*I and *Sal*I sites, but the rest of the plasmid is same as pREP41. A selection marker, the *S. cerevisiae LEU2* gene, complements *S. pombe leu*1 mutants. *ars*1 is an *S. pombe* replication origin.

2.1.11.2.2. Generation of *S. pombe pik1-td* cells

The hemizygous N1231 *pik1*/Δ*pik1*::*ura4* cells were transformed by electroporation with the expression vector pREP41X-N-degron-*pik1* as described in section 2.1.7. Random spore analysis (see section 2.1.3.) was used to select a haploid strain in which the chromosomal *pik1* coding region was replaced with the *ura4* expression cassette and that contained the episome pREP41X-Ub-R-DHFR^{ts}-*pik1*. Strain N1366 is: h$^-$ Δ*pik1*::*ura4 ade6-M216 leu1-32 ura4-D18*, pREP41X-Ub-R-DHFR^{ts}-*pik1* (referred as *pik1-td* cells).

2.1.11.2.3. *S. pombe pik1-td* cell culture

For *pik1-td* cell cultures, EMM without leucine and uracil but with 5 μM thiamine was inoculated with cells from a single colony of strain N1366 and incubated at 25^{o}C overnight. Cells from these precultures were counted, and two identical cultures started at a cell density of 1 x 10^5 cells/mL. One culture flask was incubated at 25°C for 20 – 24 hours (early to mid-logarithmic phase of growth). The other culture flask was incubated at 25°C for 12 hours, and then shifted to 36°C for another 18 hours. The incubation for 18 hours at 36°C was optimized after several trials to best observe the various cellular phenotypes since the N-degron approach is dependent upon the kinetics of *pik1* protein degradation (Rajagopalan *et al.*, 2004).

2.1.12. Yeast protein extraction

2.1.12.1. *S. pombe* protein extraction

Two methods were used for *S. pombe* protein extraction: post-alkaline extraction (section 2.1.12.2.) or small-scale extraction. The latter method was described by Moreno *et al*. (1991). Briefly, cells from a 50 mL cultures in the mid-logarithmic phase of growth were harvested at 3000 x g for 5 minutes. Cells were washed in ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3, pH8), and collected by centrifugation at 3000 x g for 5 minutes. Cells were resuspended in 20 – 50 μL of extraction buffer (10 mM sodium phosphate pH 7, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 4 μg/mL leupeptin, 1 mM PMSF) with immediate addition of protease inhibitor cocktail (100 μL per 70 mg

cell pellet) (sigma P-8215). Acid-washed glass beads 1.5 mL (0.45 – 0.50 mm diameter) were added, and the tube was vigorously vortexed for 1 minute. After the addition of 5xSDS sample buffer (250 mM Tris-HCl pH6.8, 10% SDS, 50% glycerol, 0.5 mg/mL bromophenol blue, 1.8 M β-mercaptoethanol), the samples were placed in a 100°C waterbath for 5 minutes, and the supernatants were collected by centrifugation. They were immediately used for western blot analysis or stored at -20ºC.

2.1.12.2. *S. cerevisiae* protein extraction

A post-alkaline extraction was used for *S. cerevisiae* protein extraction (Kushnirov, 2000). Cells (50 mL) were harvested at mid-log phase, and washed with ice-cold distilled water. About 100 – 200 µL of 0.2 M NaOH was added to the cell pellet, mixed, and incubated at room temperature for 5 – 12 minutes. Cells were spun down at 13000 x g for 1 minute, and the supernatant was discarded. About 50 – 100 µL of SDS sample buffer (0.06 M Tris-HCl pH6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, 0.0025% bromophenol blue) and acid-washed glass beads 100 – 200 µL (0.45 – 0.50 mm diameter) were added into the cell pellet. The cell pellet with the SDS sample buffer and glass beads was vigorously vortexed for 3 minutes, and boiled for 5 minutes. The supernatant was collected by centrifugation. It was immediately used for western Blot analysis or stored at -20ºC.

2.2. Molecular biology techniques

2.2.1. *Escherichia coli* cell cultures

E. coli DH5α [F' phi80 dlacZdeltaM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rk-,mk+) supE44 relA1 deoR delta (lacZYA-argF)U169] cells were used. Cells were cultured in LB (1% Bacto-Tryptone, 0.5% Bacto-Yeast extract, 1% NaCl) broth with or without 100 µg/mL of Ampicillin at 37^{o}C overnight. *E. coli* DH5α cells were transformed with plasmids (Table 2.4., page 77) conferring resistance to ampicillin. For LB agar plates, LB broth was solidified with 1.2% agar (W/V).

2.2.2. Electrocompetent cell preparations

On day one, *E. coli* DH5α cells were streaked on LB medium plate, and incubated at 37^{o}C overnight. On day 2, cells from a single colony were used to inoculate 10 mL of LB broth, and incubated at 37^{o}C overnight. Meanwhile, 1 L of LB medium, 1 L of sterile distilled water, and 100 mL of sterile 10% glycerol were prepared, and stored at 4^{o}C. Day three, 10 mL cultured cells were added to 1 L of LB medium, and incubated at 37^{o}C until an OD 600 nm = 0.5 - 0.7. Cells were cooled on ice for at least 15 minutes or for up to 2 hours. Cells were washed with ice-cold sterile distilled water at least three times by centrifugation at 3000 x g for 15 minutes with an SLA-1500 rotor. All cells and equipments were kept ice-cold. The last wash was with 10% glycerol (vol/vol) by centrifugation at 4300 x g for 15 minutes with an SS-34 rotor. Cells were resuspended in 1 mL of 10% glycerol, and aliquoted (25 to 50 µL/tube). The aliquots were kept at -80^{o}C untill used for transformations.

2.2.3. *E. coli* cell transformations

Electroporation was used for cell transformation. The electroporator settings were: 200 ohms resistance, 25 µFD capacitance, and 2.5 kV volts (BioRad Gene Pulser^{TM} 165-2076, USA). Up to 1 µg of plasmids in 1 – 5 µL volume, 25 – 50 µL of electrocompetent cells, and 50 µL of sterile distilled water were placed into a pre-chilled cuvette (disposable 2 mm Gap Cuvette, BTX, San Diego, USA), and gently mixed by tapping. After the pulse, 0.5 – 1 mL of SOC (2% Bacto-Tryptone, 0.5% Bacto-Yeast extract, 0.05% NaCl, and 10 mM $MgCl_2$; and 20 mM glucose added after autoclave) medium was immediately added. The cells were transferred into a culture tube, and incubated at 37^{o}C for at least 45 minutes. The transformed cells were plated on LB plate containing 100 µg/mL Ampicillin. The plates were incubated at 37^{o}C overnight until colonies formed. Colonies were restreaked on fresh media plate.

2.2.4. PCR amplification

The PCR method was used for introduction of selected changes in DNA sequence and amplification of DNA fragments. The PCR mix included the following common components: 3 µL of 10x PCR buffer (200 mM Tris-HCl pH8.4, 500 mM KCl), 0.12 µL of 25 mM dNTP, 0.15 µL of 5 U/µL Taq polymerase, 0.15 µL of each 100

ρmol/μL primer, 0.3 μL of 1 ng/μL plasmid DNA, and sterile distilled water up to a final reaction volume of 30 μL. PCR cycling programs differ in annealing temperatures and extension time, which were determined by the GC content of the primer set and the predicted PCR product length, respectively. A typical cycling program was: step 1, 95^{o}C for 1 to 5 minutes; step 2 (denaturation), 95^{o}C for 1 minute; step 3 (annealing), X^{o}C for 1 minute; step 4 (extension), 72^{o}C for Y minute(s); step 5, return to step 2 and repeat in 30 to 35 cycles; step 6, 72^{o}C for 10 minutes; step 7, keep at 4 to 6^{o}C. The annealing temperature in Celsius was determined by the equation (4x(number of Guanine + number of Cytosine) + 2x(number of Adenine + number of Thymine)) or using the NetPrimer software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html), and the extension time Y was determined by expected PCR product length (1 minute per about 1kb). Primer pairs used are demonstrated in each section, and their sequences are listed in Table 2.5.

For yeast colony PCR, the PCR reagents and cycling program were similar, but yeast cells touched with sterile micropipette tips were used instead of plasmid DNA. Sequencing was performed using service in Plant Biotechnology Institute, Saskatoon, Canada.

Table 2.5. Primers

Primers	Sequences
H1371	CGC CAG GGT TTT CCC AGT CAC GAC
H1398	GCT CTA GAG CCC CTT ATT GAT CTG ACC TT
H1399	CGG GAT CCC GAT ATT AAG TCG ATG TTG ATG C
H1400	CGG GAT CCC GAC GGA GCT TCC ACA CTT G
H1401	GGG GTA CCC CGT AAT AAT TCA CCT CAG TTG G
H1402	CGC GGA TCC CCG GGT TAA TTA A
H1403	CCG GGA TCC GAA TTC GAG CTC GTT TAA AC
H1404	AGC GGA TCC CAA TTT CAC ACA GGA
H1430	TGT TTG ACT AGT ATA ATC ACG ACT
H1436	ATC TGT GCC GAT ATC TGT TCC CCA T
H1534	CTT TTT GCT TTA CAA GAT ATA T
H1535	AAT CTT TTG CAC CTT AGC C
H1536	TGT ACA AAA TTG CTT CTT GG
H1537	CCA AGA AGC AAT TTT GTA CA
H1603	GTT GGA TCC ATA TGC CAT CTT CGA AT
H1607	TCC CCC GGG CTA GTA AAT TCC GTT CGT AAT A
H1608	CGT CCT CGA GAT GCA GAT TTT CGT CAA GAC
H1609	CGT CAG ATC TGG TAC CGT CTT TCT TCT CG
H1616	ATA AAG TCT CGT CCA AAC ACT ACA ATT AAA CTT TTG AAT TAA CAA ATC CAC AAA GTC ATC
H1619	GTT AAA TCA TAT GAG TAA AGG AGA A
H1620	GAA GAT GGC ATA TGT TTG TAT AG
H1658	TTC CAA AGA AAT ATG AAT GT
H1659	ACT ATA GTG TTG CTA AAA GT

(5´ to 3´)

2.2.5. *E. coli* plasmid DNA preparations

An alkaline lysis method was used to prepare *E. coli* plasmid DNA. For minipreparation, 1.5 mL of cells was cultured at 37^{o}C overnight. Cells were collected into an eppendorf tube by centrifugation, and the supernatant was aspirated. The bacterial cells were resuspended with 100 μL of ice-cold solution 1 (50 mM glucose, 25 mM Tris-Cl pH8.0, 10 mM EDTA pH8.0). 200 μL of alkaline lysis solution (0.2 N NaOH, 1% SDS) was added into the eppendorf tube, and were mixed by gently inverting. The eppendorf tube was kept on ice, and 150 μL of ice-cold solution 3 (3 M potassium acetate, glacial acetic acid) was added. The eppendorf tube was inverted several times, and kept on ice for 5 minutes. The bacterial cell lysate was centrifuged at maximum speed using a benchtop microcentrifuge for 5 minutes at 4^{o}C, and the supernatant was transferred into a new eppendorf tube. To precipitate plasmid DNA, two volumes of 95 - 100% ice-cold ethanol and 1/10 volume of 3 M sodium acetate were added. After inverting several times, the mixture was centrifuged at maximum speed for 10 minutes at 4^{o}C. The supernatant was aspirated, and the pellet was washed with 70% ethanol 2 – 3 times. The remaining ethanol was evaporated, and about 100 μL of TE (10 mM Tris-Cl pH8.0, 1 mM EDTA pH8.0) buffer was added to dissolve the plasmid DNA pellet. For maxipreparation, a commercial kit (Qiagen) was used.

2.2.6. Electrophoresis

2.2.6.1. Agarose electrophoresis

Agarose gel electrophoresis was performed in 0.75 to 1% agarose in TBE buffer (45 mM Tris-Borate, 1 mM EDTA). To visualize DNA fragments, the agarose gel included 0.5 μg/mL of ethidium bromide (Sambrook and Russell, 2001).

2.2.6.2. SDS-Polyacrylamide gel electrophoresis and protein band visualization

SDS-Polyacrylamide gel electrophoresis (PAGE) was performed as described in Sambrook and Russell (2001). 6% or 10% separating gels containing 30% acrylamide (37.5 : 1 ratio of acrylamide : bisacrylamide) were used to separate proteins 90 – 220 kDa or proteins 40 – 90 kDa, respectively. Stacking gels containing 5% acrylamide / bisacrylamide were used. A Mini-PROTEIN II Cell (BioRad 165-2940, Hercules, USA)

was used with the conditions for running the gel: 150 volts, 60 mA per gel, and 1–1.5 hours. Protein molecular weight markers used for SDS-PAGE were purchased from Invitrogen, Burlington, Canada.

Protein bands in SDS-Polyacrylamide gels were visualized by Coomassie Brilliant Blue or silver staining. For Coomassie Brilliant Blue staining, an SDS-Polyacrylamide gel was soaked in the staining solution (0.05% Coomassie Brilliant Blue R-250, 25% isopropanol, 10% acetic acid), and agitated for 10 – 15 minutes. The gel was rinsed twice with distilled water, and destained with 10% acetic acid solution. A commercial silver staining kit (Amersham Biosciences kit 17-1150-01, Piscataway, USA) was used. The stained gels were air-dried in cellophane (BioRad 1651779).

2.2.7. Western blot analysis

The proteins in the SDS-Polyacrylamide gel were transferred onto 0.45 μm nitrocellulose (Fisher Scientific Co. EP4HY00010) using a Mini Trans-Blot electrophoretic Transfer kit (BioRad 170-3937 and 170-3935). To visualize the effectiveness of protein transfer, the nitrocellulose was staining with ponceau S solution (Sigma P-7170). The ponceau S solution was then washed away with distilled water, and the nitrocellulose sheet was then used for western blot analysis. Western blot analysis was described in Sambrook and Russell (2001): step 1, primary blocking with 5% skim milk in TNE (10 mM Tris-HCl pH7.5, 2.5 mM EDTA pH8.0, 50 mM NaCl, 0.05% tween 20) buffer at room temperature for 30 minutes to 1 hour with gentle shaking; step 2, primary antibody (1:1000 dilution) reaction at room temperature for 1 hour or at 4^{o}C overnight with gentle shaking; step 3, washing with TNE buffer 5 times for 5 minutes each with gentle shaking; step 4, secondary blocking 5% skim milk in TNE buffer for 30 minutes with gentle shaking; step 5, peroxidase-conjugated secondary antibody (1:5000 dilution) reaction at room temperature for 1 hour with gentle shaking; step 6, washing with TNE buffer 5 times for 5 – 10 minutes each time with gentle shaking; step 7, detection using a commercial horseradish peroxidase (HRP) – chemiluminescent detection kit (ECL kit; Amersham Biosciences RPN2132) and imaging films (Kodak 1651454).

2.3. Identification of Pik1p – protein interactions

Protein purification will vary depending on biochemical characteristics of the protein of interest. A tandem-affinity-purification (TAP) approach is very attractive because its generic purification steps allow the purification of proteins, which will have different biochemical characteristics. Also, this approach is speculated to less disturb structures and/or interactions of proteins. Two stringent affinity-steps will increase the purity and recovery rate of proteins. A first affinity purification is achieved by the affinity of an IgG binding domain from *Staphylococcus aureus* Protein A (Prot A) to IgG sepharose. A second affinity purification is achieved by the affinity of a calmodulin-binding domain (CBD) to calmodulin resins in the presence of calcium ions. To purify proteins interacting with Pik1p from *S. pombe* cells, a TAP approach was used.

2.3.1. Construction of plasmid, pREP41-NTAP-*pik1*

A plasmid pREP41-NTAP was generously provided by K. Gould (Vandervilt University, Nashville, USA) (Tasto *et al.*, 2001). The *S. pombe pik1* cDNA sequence which possesses a silent mutation (see section 2.1.8.) was cloned between the *Nde*I and *Bam*HI sites of a plasmid pREP41-NTAP, resulting in an episome, pREP41-NTAP-*pik1*. The plasmid pREP41-NTAP-*pik1* was introduced into the hemizygous diploid cells (strain N1231) using a lithium acetate method (section 2.1.4.1.1.). A random spore analysis (section 2.1.3.) was performed to isolate haploid cells in which the genomic *pik1* coding region was deleted and contained the episome pREP41-NTAP-*pik1* (strain N1240). Wild-type haploid cells (strain N3) were transformed with plasmid pREP41-NTAP (strain N1361) as a control.

2.3.2. Tandem-affinity-purification (TAP) tag – Pik1 protein purification

The purification process was described in Gould *et al*. (2004). Briefly, 2 L cultures of each *S. pombe* strain, N1240 and N1361, were grown to mid-logarithmic phase of growth (OD_{595} = 0.5-0.7). Cells were collected by centrifugation and the supernatant was removed. To prepare a protein lysate, a cell lysis buffer containing NP-40 (6 mM Na_2HPO_4, 4 mM $NaH_2PO_4H_2O$, 1.5% NONIDET P-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 μg/mL leupeptin, 0.1 mM Na_3VO_4, 1.3 mM Benzamidine, 0.3

mM PMSF, complete protease inhibitor tablet) was added. A cell lysate was prepared using a French Press at 11000 psi (AMONCO 4-3339, USA) 4 – 5 times. To remove the cell debris, the cell lysate was centrifuged at 4^{o}C, 1500 x g (Sorvall SLA-1500) for 5 minutes. The supernatants were transferred into new 50-mL tubes, and 800 µL of IgG Sepharose beads in NP-40 buffer was added. The mixtures were agitated at 4^{o}C for 2 hours. The beads were compacted by gravity at 4^{o}C in a column, and washed 3 times with a washing buffer I (10 mM Tris-HCl pH8.0, 150 mM NaCl, 0.1% NP-40). The fourth wash was performed with freshly prepared Tabacco Etch Virus (TEV) protease cleavage buffer (10 mM Tris-HCl pH8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1.0 mM DTT). Then, 1 mL of TEV cleavage buffer and 300 – 400 U of TEV were added into the column containing the beads, and the mixtures were incubated at 16^{o}C for 2 hours with agitation. The eluate was collected into a new column. The three volumes of 0.1% NP-40 calmodulin binding buffer (0.1% NP-40, 10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM Mg^{2+}acetate, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol), 3 µL of 1 M $CaCl_2$ per mL IgG eluate, and 300 µL of calmodulin resin in 0.1% NP-40 calmodulin binding buffer were added in the new column containing the eluate. The mixtures were incubated with agitation at 4^{o}C for 1.5 hours. The resins were washed twice with 1 mL of 0.1% NP-40 calmodulin binding buffer, and a third wash was performed with 1 mL of 0.02% NP-40 calmodulin binding buffer (0.02% NP-40, 10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM Mg^{2+}acetate, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol). The elution was performed by adding 2 – 3 mL of an elution buffer (10 mM Tris-HCl pH8.0, 150 mM NaCl, 0.02% NP-40, 1 mM Mg^{2+}acetate, 1 mM imidazole, 20 mM EGTA, 10 mM β-mercaptoethanol). Proteins in the eluate were precipitated with 25% TCA (trichloroacetic acid) as described (Gould *et al.*, 2004). The precipitated proteins were dried and stored at -20^{o}C.

2.3.3. Mass spectrometry (MS) analysis of TAP-Pik1 protein complexes

The TCA precipitated protein preparations used for MS analysis were as described (Link *et al.*, 1999). Samples were sent to J. Yates in Yeast Resource Center, University of Washington, Seattle, USA. The TCA-precipitated protein samples were subjected to proteolysis, and the resulting peptides separated by multidimensional liquid

chromatography and tandem MS. The SEQUEST algorithm was used to infer amino acid sequences.

2.4. Microscopy

2.4.1. Fixation of *S. pombe* cells with formaldehyde

Formaldehyde fixation of *S. pombe* cells was as described (Moreno *et al.*, 1991). Formaldehyde (17.5%) was freshly prepared by adding 8.75 g of paraformaldehyde to 50 mL PBS (10 mM Na_2HPO_4, 150 mM NaCl) buffer and 1 mL of 1 M NaOH. Formaldehyde was dissolved by incubation at 65^{o}C for 15 – 20 minutes followed by centrifugation at 2500 x g for 5 minutes to remove undissolved polymers. 10 mL of freshly prepared formaldehyde (10 mL) was added to 40 mL cell cultures at mid-logarithmic phase of growth. Incubation was for 30 minute on a rotating wheel. After spinning cells down at 2500 x g for 5 minutes, cells were washed three times with PBS buffer. The formaldehyde-fixed cells were stored at 4^{o}C in PBS buffer containing 1 mM NaN_3 for a maximum of 1 to 2 weeks.

2.4.2. Fixation of *S. pombe* cells with methanol

Methanol fixation of *S. pombe* cells was as described (Moreno *et al.*, 1991). 10 mL of culture medium with cells at mid-logarithmic phase of growth was filtered using a vacuum driven 0.22 μm disposable bottle top filter (Millipore SCGPT01RE, MA, USA). The cells were washed once with precooled 100% methanol, and about 30 mL of 100% methanol was added to the cells in the bottle top filter. These cells were kept at -20^{o}C for 10 minutes. The cell flakes were scaped off the filter membrane and collected by centrifugation at 2500 x g for 5 minutes. The cell pellets were incubated for 5 minutes each with 75, 50, and 25% methanol in PBS buffer sequentially with gentle shaking. The last incubation was done in PBS buffer without methanol. The methanol-fixed cells were stored at 4^{o}C in PBS buffer containing 1 mM NaN_3.

2.4.3. Cell length measurement

Bright-field or fluorescence microscopy was with an Olympus 1X70 inverted microscope with 60X 1.4NA Plan-apo objective, appropriate filter sets and a RT-Slider

(SPOT) CCD camera (Carsen Scientific Imaging Group, Markham, Canada). Images were cropped and processed for brightness and contrast with Spot32 Advanced software. Cell lengths were estimated relative to a micrometer bar.

2.4.4. Visualization of F-actin structures with FITC-conjugated phalloidin

Formaldehyde-fixed cells (20-50 µL) were mixed with 5 µL of 0.1 mg/mL FITC-conjugated phalloidin in PBS buffer. The mixture was incubated for 30 minutes to 1 hour on a rotating wheel. The mixture was washed 1 – 2 times with PBS buffer by centrifugation at 720 – 1000 x g for 1 – 2 minutes. The cells were mounted on a coverslip with 10 µL of DAPI-containing mounting solution (1 mg/mL ρ-phenylenediamine, 50% glycerol, 1 µg/mL DAPI, 1 mM NaN_3 in PBS buffer). See section 2.4.3.2. for DAPI staining.

2.4.5. Visualization of nucleus and septum with DAPI and Calcofluor white

Formaldehyde-fixed cells (50 uL) were mixed with 450 µL of PBS buffer and 2.5 µL of 10 mg/mL Calcofluor white stock solution. The mixture was incubated for 15 minutes on a rotating wheel. The cells were washed 5 times with PBS buffer. These cells were immobilized on a poly(L-lysine)-coated coverslip (Moreno *et al.*, 1991) with 10 µL of DAPI mounting solution.

2.4.6. Indirect immunofluorescence microscopy of Pik1p

Pik1p immunofluorescence microscopy of methanol-fixed cells was done with primary antibodies and a Texas red conjugated secondary antibody at a 1:100 dilution as described (Moreno *et al.*, 1991). Polyclonal rabbit antibodies against Pik1p were available from previous work in this laboratory (Desautels *et al.*, 2001). Briefly, methanol-fixed cell walls were digested with 0.25 mg/mL Zymolyase 20T (ICN 320921, Ohio, USA) and 0.25 mg/mL Lysing enzyme (Sigma L-2265) in PBS buffer containing 1.2 M sorbitol. The mixtures were incubated for 15 minutes, and equivalent volume of PBS buffer containing 2% Triton X-100 was immediately added. Cells were centrifuged at 1000 x g for 3 minutes, and washed 5 times with PBS buffer. To reduce unspecific binding, 1 mL of PBAL (PBS buffer containing 100 mM lysine-HCl and 1% fatty acid-

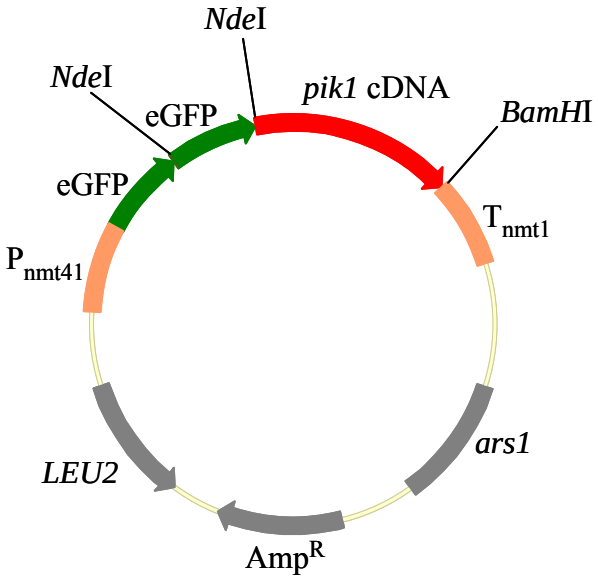
free bovine serum albumin) solution was added into the cell pellets. After resuspention, the mixture was incubated for 30 minutes on a rotating wheel. For the primary antibody reaction, 5 μL of polyclonal rabbit antibodies against Pik1p and 500 μL of PBAL solution were added into the cell pellets, and the cells were incubated overnight on a rotating wheel. The cells were washed 4 times with PBAL solution, and the fifth wash was for 20 minutes. Then, 5 μL of anti-rabbit Texas-red and 500 μL of PBAL solution were added into the cell pellets, and the cells were resuspended and incubated for 1 hour on a rotating wheel in the dark. The cells were washed 4 times with PBS buffer, and the fifth wash was for 20 minutes. The cells were immobilized on a poly(L-lysine)-coated coverslip with 10 μL of DAPI mounting solution.

2.4.7. Fluorescence microscopy of cells expressing a gene encoding a 2XeGFP-Pik1 fusion protein

To assess the subcellular localization(s) of *S. pombe* Pik1p in live cells, ectopic expression of an enhanced green fluorescent protein (eGFP) fused to *pik1* tagging was used first in asynchronous cultures and then in *S. pombe cdc25-22* cells synchronized by temperature block and release from G2/M (as described in section 2.1.2.2.).

2.4.7.1. Construction of plasmid, pREP41-2XeGFP-*pik1*

An *Nde*I – *Bam*HI fragment of *pik1* cDNA was cloned at the 3´-end of the eGFP coding sequence in pREP41-eGFP (Craven *et al.*, 1998). A second eGFP gene with *Nde*I sites at both its 5´ and 3´ ends was amplified and inserted immediately 5´of the *pik1* gene, to allow expression of a 2XeGFP-Pik1 fusion protein under the control of an attenuated *nmt1* promoter. To amplify the eGFP gene from a plasmid pREP41-eGFP with *Nde*I sites, a primer set H1619/H1620 was used (Figure 2.4.). The ligation direction of the second eGFP was confirmed by restriction enzyme digestion.



pREP41-2XeGFP-*pik1*

Figure 2.4. Plasmid pREP41-2XeGFP-*pik1* for cellular localization study. The original plasmid used was pREP41-eGFP. *pik1* cDNA sequences were first cloned between *Nde*I and *Bam*HI site, and eGFP coding sequences were then cloned into the *Nde*I site.

2.4.7.2. Visualization of 2XeGFP-Pik1 fusion proteins

Transformation of the hemizygous strain N1231 was followed by random spore analysis to select a haploid strain in which the chromosomal *pik1* gene was replaced with the *ura4* expression cassette but remained viable from episomal *pik1* expression. Strain N1369 is: h^+ Δ*pik1*::*ura4 ade6-M210 leu1-32 ura4-D18*, pREP41-2XeGFP-*pik1*. The N1369 cell cultures were started at a cell density of 1 x 10^5 cells/mL in the presence or absence of thiamine from an overnight pre-culture at 30°C in the presence of thiamine, and incubated for 20 – 24 hours at 30°C. The culture medium for the N1369 cells was minimal medium EMM lacking leucine and uracil in the presence or absence of 5 μM thiamine. The live cells of the strain N1369 were observed under a fluorescence microscope.

Transformation of a strain carrying the *cdc25-22* allele was followed by selection of cell viability in EMM media lacking leucine. Strain N1401 is: h^- *cdc25-22 leu*1-32, pREP41-2XeGFP-*pik1*. To synchronize cell cultures, cells of the strain N1401 were grown at 25°C in EMM lacking leucine for 16 hours in the absence of thiamine, to allow some expression of the fusion protein. The cells were then incubated for 4 hours at 36°C and collected by centrifugation (5 minutes at 3000 x g). Medium (50 mL), pre-warmed to 25°C, was added and the cultures were incubated for another 220 minutes with two aliquots (0.8 mL per aliquot) taken every 20 minutes. One aliquot was used to fix the cells by adding 0.2 mL formaldehyde (Moreno *et al.*, 1991). These cells were incubated for 30 minutes on a rotating wheel, washed 3 times in PBS buffer and kept at 4°C, and stored until microscopic examination. The other aliquot was used to evaluate the localization of 2xEGFP-Pik1p immediately in live cells.

2.4.8. Transmission electron microscopy

Transmission electron microscopy was performed with potassium permanganate-fixed *pik1-td* cells as described (Armstrong *et al.*, 1993; Wright, 2000; Wang *et al.*, 2002). The *pik1-td* cells were cultured as described in section 2.1.7.2.3. Cell preparation for transmission electron microscopy and visualization of intracellular cell structures was performed in collaboration with P.A. Netto in the Temasek Life Sciences Laboratory, Singapore. Transmission electron microscopy was performed by P.A.Netto.

Chapter 3: Results

3.1. Heterologous expression of *S. pombe pik1* in *S. cerevisiae pik1-101* and *PIK1*

The *S. cerevisiae pik1* orthologue, *PIK1*, has been biochemically and physiologically studied. *PIK1* encodes a type IIIβ PtdIns 4-kinase of 1066 amino acids (Flanagan and Thorner, 1992; Flanagan *et al.*, 1993; Garcia-Bustos *et al.*, 1994). The *S. cerevisiae pik1-101* allele has a point mutation that changes a residue ($1045^{Ser \rightarrow Phe}$) in the C-terminal catalytic domain. The *pik1-101* allele is conditionally lethal with a restrictive temperature of 37°C (Walch-Solimena and Novick, 1999). Mutant cells become enlarged and multinucleate at 37°C. Furthermore, the distribution of F actin becomes disrupted in *pik1-101* cells at 37°C. The lipid kinase activity was determined through mixing immunoprecipitated Pik1p or Pik1-101p with a substrate PtdIns *in vitro* (Walch-Solimena and Novick, 1999). The lipid kinase activity of Pik1-101p was reduced at 37°C and even at a permissive temperature, 25°C. However, the reduction was greater at 37°C than at 25°C.

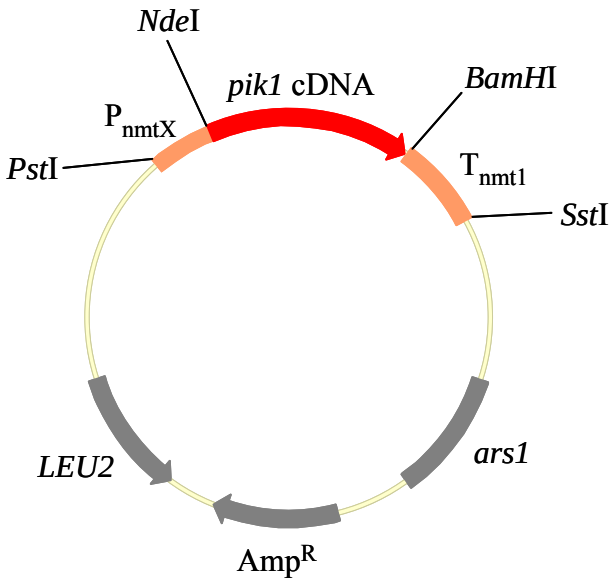
PIK1 is an essential gene, indicating that it has one or more essential functions that are not provided by the two other PtdIns 4-kinases present in *S. cerevisiae* (Stt4p and Lsb6p). Comparison of the sequences of *S. cerevisiae PIK1* and *S. pombe pik1* suggests that the latter also encodes a type IIIβ PtdIns 4-kinase (Desautels *et al.*, 2001). Whether the essential functions of *S. cerevisiae PIK1* are conserved in *S. pombe pik1* is assessed in this section.

3.1.1. Expression of *S. pombe pik1* restores the defective growth of *S. cerevisiae pik1-101* at the restrictive temperature

An approach to determining if *S. pombe pik1* can provide the essential function(s) of *S. cerevisiae PIK1* would be to express *S. pombe pik1* in *S. cerevisiae pik1-101* cells and to examine cell viability and colony formation at 37°C. However, *S. pombe* cells are highly sensitive to changes in *pik1* expression; for example, ectopic expression of *pik1* cDNA sequences under the control of a plasmid-born, wild-type *nmt1* (*n*o *m*essage in *t*hiamine) promoter caused dominant lethality with high penetrance (Steinbach *et al*., unpublished). If *S. cerevisiae* cells are similarly sensitive to changes in *PIK1* expression, and if *S. pombe* Pik1p is active and capable of providing the essential functions of Pik1p in *S. cerevisiae*, then improperly regulated expression of *pik1* might result in lethality, thus confounding the experimental approach. In *S. pombe*, ectopic expression of *pik1* under the control of an attenuated version of the *nmt1* promoter, such as that present on pREP41, fully complemented deletion of the chromosomal locus (Park *et al*., unpublished). I therefore wished to test *pik1* expression in *S. cerevisiae pik1-101* cells under the control of promoters of varying strengths. A series of expression cassettes had been previously constructed (Figure 3.1., page 89) (Steinbach *et al*., unpublished), in which the *S. pombe pik1* coding region, as the cDNA sequence, was inserted between 3 different versions of the *S. pombe nmt1* promoter and the *nmt1* terminator sequences. The promoter versions included the wild-type promoter sequence, and an attenuated and a highly attenuated version of the promoter: P_{nmt1}, P_{nmt41} and P_{nmt81}, respectively (Basi *et al.*, 1993). The promoters are repressed in the presence of thiamine, but they are derepressed in the absence of thiamine (Basi *et al.*, 1993). These promoter variants provide a range of expression levels in *S. pombe* (Forsburg, 1993). Each expression cassette was inserted into the *S. cerevisiae* plasmid vector, YEplac181 (Gietz and Sugino, 1988) to produce the following expression vectors (Figure 2.1. A): YEplac181-P_{nmt1}-*S. pombe pik1*-T_{nmt1}, YEplac181-P_{nmt41}-*S. pombe pik1*-T_{nmt1}, and YEplac181-P_{nmt81}-*S. pombe pik1*-T_{nmt1}. *S. cerevisiae pik1-101* cells were transformed with each plasmid as described in Materials and Methods. *S. cerevisiae pik1-101* cells carrying any of these plasmids were able to form colonies at 25°C regardless of the presence or absence of exogenous thiamine (Figure 3.2. A, page 91). *S. cerevisiae pik - 101* cells carrying YEplac181 failed to form colonies at 37°C regardless of the presence or absence of exogenous thiamine (Figure 3.2. A). The cells were enlarged and

multinucleate and frequently had 1 or 2 attached daughter cells as the phenotype of *S. cerevisiae pik1-101* cells was previously observed (Walch-Solimena and Novick, 1999). Expression of *S. pombe pik1* cDNA under the control of P_{nmt1} supported some colony formation by *S. cerevisiae pik1-101* cells at 37°C (Figure 3.2. A). Use of the attenuated promoter supported a reduced level of colony formation compared to the wild-type promoter and colony formation was not observed at 37°C when the highly attenuated promoter was used. These results indicate that *S. pombe pik1* can provide essential functions of Pik1p in an *S. cerevisiae* loss-of-function mutant and that *S. pombe nmt1* promoter sequences are useful in *S. cerevisiae*.

S. cerevisiae Pik1p is present in the Golgi apparatus and the nucleus (Walch-Solimena and Novick, 1999; Strahl *et al.*, 2005). To determine if *S. pombe* Pik1p is present in these locations in *S. cerevisiae pik1-101* cells, a gene encoding *S. pombe pik1* tagged with an enhanced green fluorescence protein (eGFP) (Figure 2.1. A, page 63) was expressed in *S. cerevisiae pik1-101* cells (Figure 3.2. B). The fluorescent signal of eGFP-Pik1p was not detected in *S. cerevisiae pik1-101* cells, possibly because of lack of efficient sensitivity of the epifluorescent and image capture system used. Surprisingly however, under the control of the attenuated *S. pombe* P_{nmt41} promoter, expression of the eGFP-*pik1* fusion gene fully restored the growth defect of *S. cerevisiae pik1-101* cells at 37°C regardless of the presence or absence of thiamine (Figure 3.2. B). Under the control of the same attenuated promoter, *S. pombe pik1* cDNA produced only a very low level of growth restoration (Figure 3.2. B). The extent to which colony formation was supported by expression of the *S. pombe pik1* cDNA was very similar in this experiment to that observed in the independent experiment shown in Figure 3.2. A. In summary, expression of a gene encoding an eGFP-Pik1p fusion produced full complementation of *S. cerevisiae pik1-101* at 37°C; whereas, expression of a gene encoding Pik1p alone produced only partial complementation. The degree to which expression of the gene encoding Pik1p alone complemented *pik1-101* was positively correlated to the strength of the version of the *nmt1* promoter that controlled the gene.

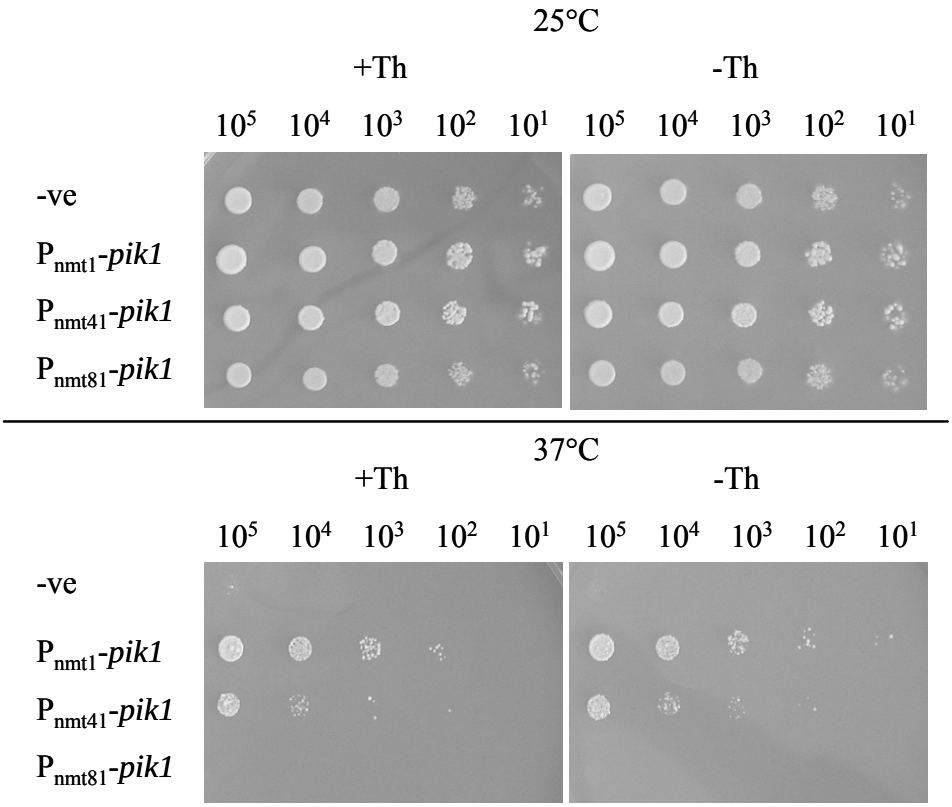


pREPx-*pik1*

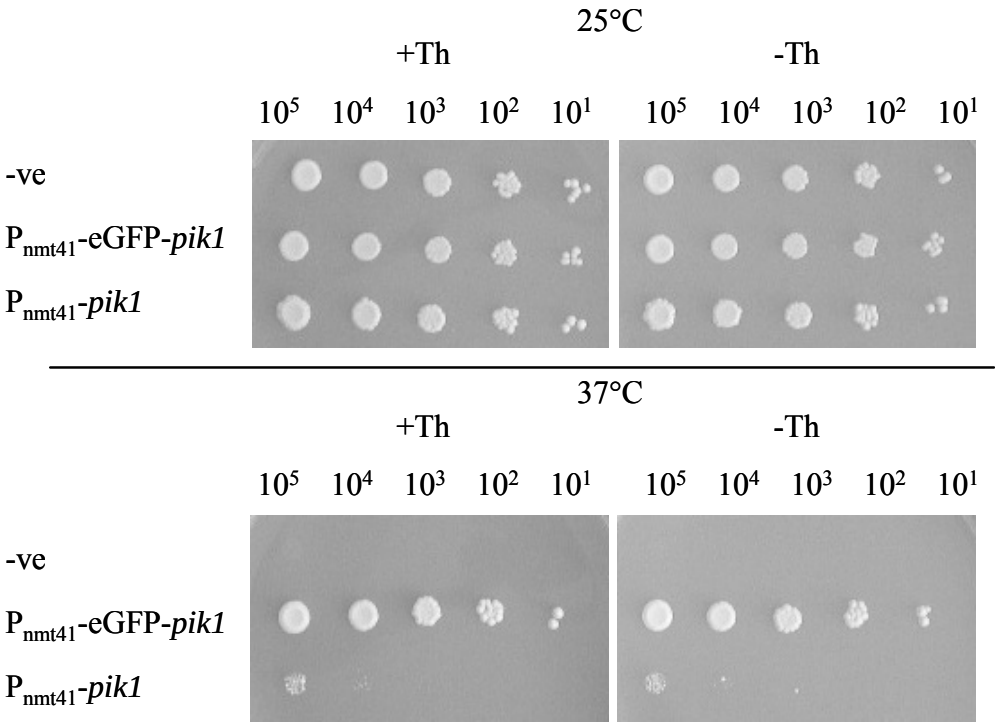
Figure 3.1. Plasmids for expression of *pik1* sequences in *S. pombe*. These plasmids were provided by S. Steinbach. The *pik1* cDNA coding region (see 2.1.9.1.) was placed under the control of the *nmt*1 promoter or an attenuated or highly attenuated version of the *nmt*1 promoter in vectors pREP1, pREP41 or pREP81, respectively. The *nmt*1 transcription terminator sequences were present in each case. The *Pst*I and *Sst*I restriction sites were used to mobilize the expression cassettes for introduction into the *S. cerevisiae* vector YEplac181 for use in complementation studies (see Figure 2.1.).

Figure 3.2. Colony formation assays: Heterologous expression of wild-type *S. pombe pik1* complements the *S. cerevisiae pik1-101* allele at the restrictive temperature. *S. cerevisiae* cells carrying a temperature-sensitive lethal allele (*pik1-101*) were transformed with plasmids as indicated below and grown in liquid culture overnight in the presence of thiamine at the permissive (25°C) temperature. To assay for colony formation at 25°C or the restrictive temperature 37°C for this allele, aliquots from serial dilutions of each culture, containing the number of cells indicated, were prepared and spotted onto SD-leucine plates with or without thiamine (+Th, -Th, respectively). Plates were then incubated for 5 days at the temperatures indicated. The colony formation assays were replicated independently at least three times. The results shown are representative of each of the replicates. (A) *S. cerevisiae pik1-101* cells were transformed with YEplac181 as negative control (-ve) or with YEplac181 recombinants that contained expression cassettes in which the *S. pombe pik1* coding region, as the cDNA sequence, was under the control of the wild-type *S. pombe nmt*1 promoter sequence (P_{nmt1}-*pik1*) or by an attenuated (P_{nmt41}-*pik1*) or highly attenuated (P_{nmt81}-*pik1*) version of that promoter. (B) *S. cerevisiae pik1-101* cells were transformed with YEplac181 as negative control (-ve) or with YEplac181 recombinants that contained a recombinant sequence encoding an eGFP-Pik1p fusion under the control of P_{nmt41} (P_{nmt41}-eGFP-*pik1*) or encoding Pik1p under the control of P_{nmt41} (P_{nmt41}-*pik1*).

A


25°C
+Th
-Th
10⁵ 10⁴ 10³ 10² 10¹ 10⁵ 10⁴ 10³ 10² 10¹
-ve
Pnmt1-pik1
Pnmt41-pik1
Pnmt81-pik1
37°C
+Th
-Th
10⁵ 10⁴ 10³ 10² 10¹ 10⁵ 10⁴ 10³ 10² 10¹
-ve
Pnmt1-pik1
Pnmt41-pik1
Pnmt81-pik1

B


25°C
+Th
-Th
10⁵ 10⁴ 10³ 10² 10¹ 10⁵ 10⁴ 10³ 10² 10¹
-ve
Pnmt41-eGFP-pik1
Pnmt41-pik1
37°C
+Th
-Th
10⁵ 10⁴ 10³ 10² 10¹ 10⁵ 10⁴ 10³ 10² 10¹
-ve
Pnmt41-eGFP-pik1
Pnmt41-pik1

3.1.2. Expression of *S. pombe pik1*^{D709A} fails to restore the defective growth of *S. cerevisiae pik1-101* cells at the restrictive temperature

A residue of the *S. pombe* Pik1p protein was identified as a potentially essential residue for its lipid kinase activity (Steinbach *et al*., unpublished). Alignment of the Pik1p primary structure with the sequences of well characterized PtdIns 4-kinase and 3-kinase enzymes (Gehrmann and Heilmeyer, Jr., 1998; Desautels *et al.*, 2001) led to the identification of D709 as a conserved residue that is required for the activities of lipid kinases. A D709A mutation was generated by Steinbach in a *pik1* cDNA sequence by site directed mutagenesis. Lipid kinase activity in crude extracts of *S. pombe* cells carrying plasmid-borne *pik1* or *pik1*^{D709A} sequences controlled by the wild-type *nmt1* promoter was measured (Steinbach *et al*., unpublished). Derepression of the plasmid-borne *pik1* gene resulted in a marked increase in lipid kinase activity. In contrast, no increase was observed upon derepression of the *pik1*^{D709A} allele (Steinbach *et al*., unpublished).

To determine whether the *S. pombe* D709 residue is required for complementation of the *S. cerevisiae pik1-101* allele, the D709A mutation was introduced into the eGFP-*pik1* fusion gene, which was under the control of the attenuated *nmt1* promoter, P_{nmt41}. *S. cerevisiae pik1-101* cells were transformed with the resulting expression vector (Figure 2.1. B, page 63). At 25°C, transformants were able to form colonies in both the presence and absence of added thiamine (Figure 3.3.). As reported in the previous section, at 37°C *S. cerevisiae pik1-101* cells carrying the vector YEplac181 failed to form colonies, whereas expression of eGFP-*pik1* completely complemented the colony formation defect of *pik1-101* (Figure 3.3.). Expression of the eGFP-*pik1*^{D709A} construct failed to support colony formation at 37°C (Figure 3.3.). The failure of D709A mutant to complement the *pik1-101* allele was observed regardless of the presence or absence of thiamine (Figure 3.3.). This indicates that the *S. pombe* Pik1p D709 residue is required for complementation.

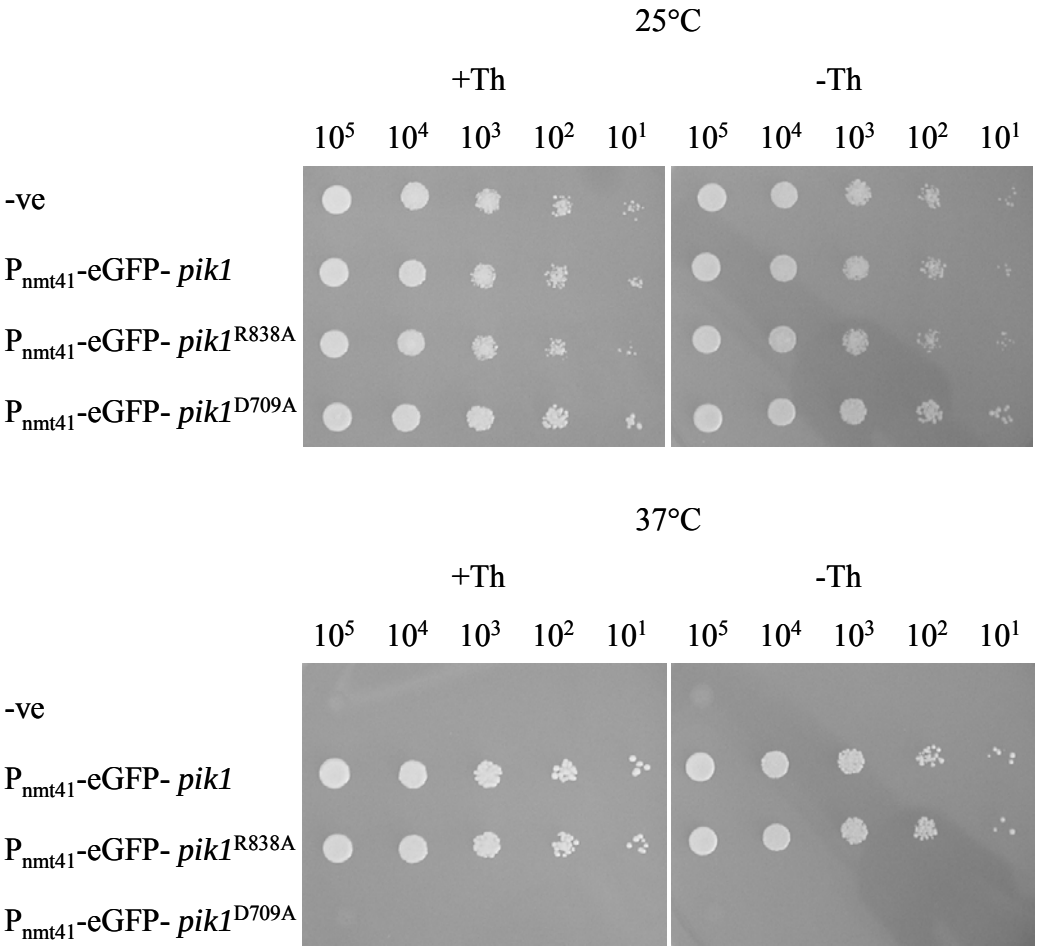


Figure 3.3. Colony formation assays: Heterologous expressions of eGFP fused *S. pombe pik1* alleles, eGFP-Pik1 and eGFP-Pik1^{R838A}, complement the *S. cerevisiae pik1-101* allele at the restrictive temperature. Cell cultures as well as the *S. cerevisiae pik1-101* transformations were carried out as described in Figure 3.2. except different plasmids were used. *S. cerevisiae pik1-101* cells were transformed with YEplac181 as negative control (-ve) or with YEplac181 recombinants that contained a recombinant sequence encoding an eGFP-Pik1p fusion under the control of P_{nmt41} (P_{nmt41}-eGFP-*pik1*), or two allelic mutants of *pik1* encoding eGFP-Pik1^{D709A} or eGFP-Pik1^{R838A} under the control of P_{nmt41} (P_{nmt41}-eGFP-*pik1*^{D709A} or P_{nmt41}-eGFP-*pik1*^{R838A}, respectively).

3.1.3. Expression of *S. pombe pik1*^{R838A} mutant allele restores the defective growth of *S. cerevisiae pik1-101* at the restrictive temperature

S. pombe Cdc4p, a contractile ring protein essential for cytokinesis (McCollum *et al.*, 1995), interacts with *S. pombe* Pik1p in yeast two-hybrid and ELISA assays (Desautels *et al.*, 2001). This interaction has not been reported to occur in any other organism. The C-terminal end of Pik1p is required for the interaction with Cdc4p in the yeast two-hybrid assay and within this region is a sequence that resembles the IQ motif (Desautels *et al.*, 2001). A mutation within the IQ motif, which is near the C-terminal end of Pik1p (R838A), abolished the interaction of Pik1p with Cdc4p in both assays (Steinbach *et al*., submitted). To determine whether the *S. pombe* R838 residue is required for complementation, the R838A mutation was introduced into the eGFP-*pik1* fusion gene which was under the control of the attenuated *nmt1* promoter, P_{nmt41}. *S. cerevisiae pik1-101* cells were transformed with the resulting expression vector (Figure 2.1. B, page 63). Transformants were able to form colonies at 25°C in the presence or absence of added thiamine. Unlike the D709A mutant, the R838A mutation was innocuous, resulting in colony formation in *S. cerevisiae pik1-101* cells at 37°C regardless of the presence or absence of exogenous thiamine (Figure 3.3., page 93).

3.1.4. Expression of wild-type *S. pombe pik1* suppresses colony formation in wild-type *S. cerevisiae* cells

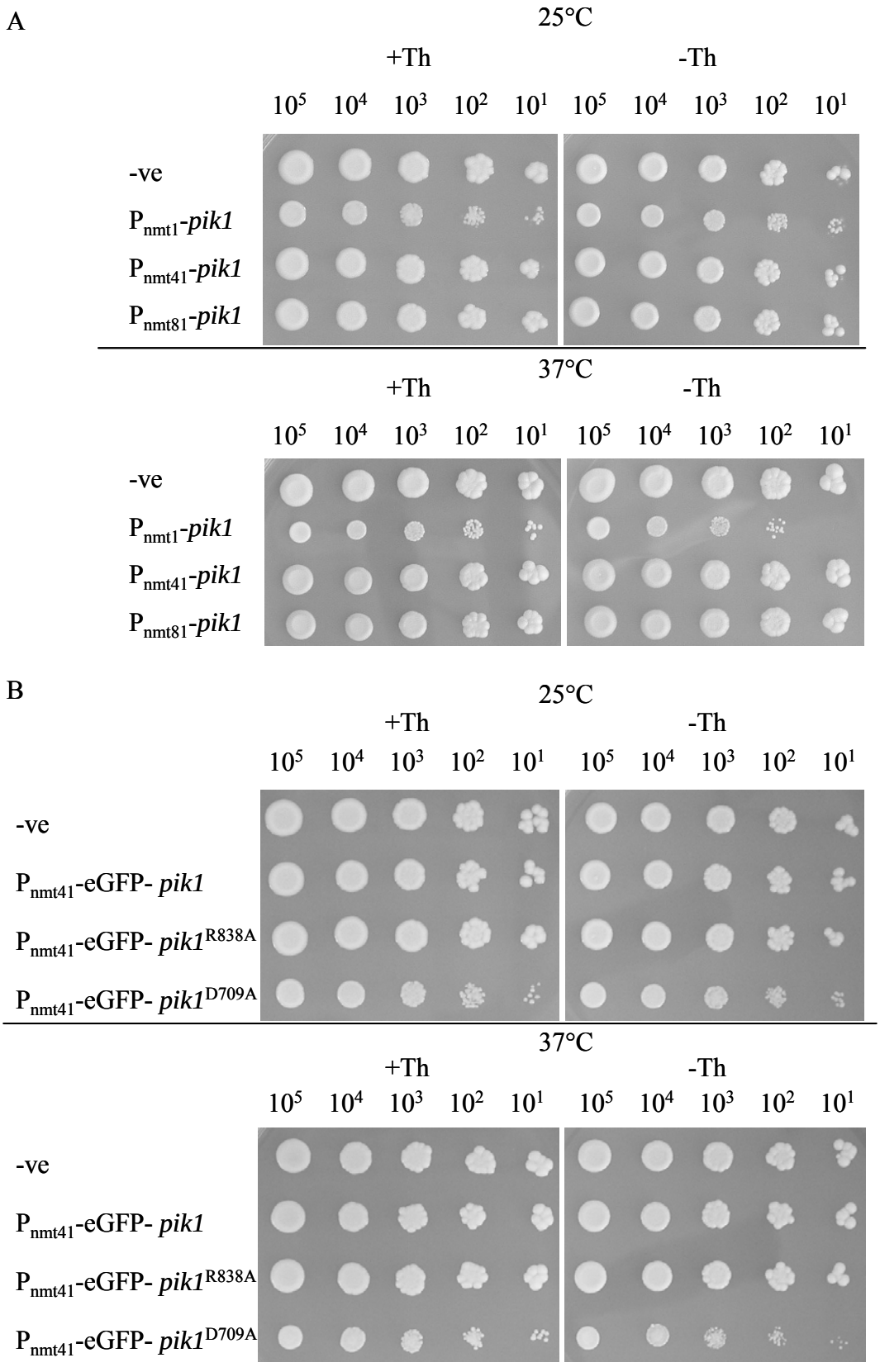
Expression of a plasmid-borne *S. pombe pik1* cDNA sequence under the control of the *nmt1* promoter in *S. pombe* cells that are wild-type for the chromosomal *pik1* locus rapidly blocks cell proliferation and results in lethality (Steinbach *et al*., unpublished). I wished to determine if heterologous expression of *S. pombe pik1* in wild-type *S. cerevisiae PIK1* cells might produce a similar effect. I assessed the ability of *S. cerevisiae PIK1* cells carrying the plasmids, YEplac181-P_{nmt1}-*S. pombe pik1*-T_{nmt1}, YEplac181-P_{nmt41}-*S. pombe pik1*-T_{nmt1}, or YEplac181-P_{nmt81}-*S. pombe pik1*-T_{nmt1}, to form colonies at 25°C and 37°C. Expression of *S. pombe pik1* cDNA under the control of P_{nmt1} suppressed colony formation in wild-type *S. cerevisiae PIK1* cells at 37°C (Figure 3.4. A, page 97). This result was shown in the presence or absence of thiamine at

both temperatures (Figure 3.4. A). This effect was not observed with the use of the attenuated promoters (Figure 3.4. A).

3.1.5. Expression of *S. pombe pik1* D709A mutant suppresses colony formation in wild-type *S. cerevisiae* cells

Expression of kinase-dead alleles of protein kinases in cells that are otherwise wild-type has been used to generate dominant negative phenotypes for the purpose of learning about the normal activities of the kinase of interest (Schmidt *et al.*, 1996). A dominant negative phenotype is dominant in that the phenotype is expressed in the presence of the wild-type allele and negative in that the outcome is deleterious for the cell. To assess whether heterologous expression of *S. pombe pik1*, *pik1*^{D709A}, or *pik1*^{R838A} causes any dominant phenotype in wild-type *S. cerevisiae PIK1* cells, the eGFP-tagged *S. pombe pik1*, *pik1*^{D709A}, or *pik1*^{R838A} alleles were expressed under the control of the attenuated promoter, P_{nmt41}, in the presence or absence of thiamine. Cells expressing the eGFP-*pik1*^{D709A} allele slowed growth at both temperatures assayed as compared to control cells that lacked the *pik1* sequences, cells expressing the eGFP-*pik1* allele, or cells expressing the eGFP-*pik1*^{R838A} allele at both temperatures regardless of the presence or absence of thiamine (Figure 3.4. B, page 97). This result demonstrates that the ectopic expression of the *S. pombe* Pik1^{D709A} kinase-dead allele generates dominant negative phenotypes in *S. cerevisiae* wild-type cells like other lipid kinases (Schmidt *et al.*, 1996).

Figure 3.4. Colony formation assays: *S. pombe pik1* expression in wild-type *S. cerevisiae* cells. Wild-type *S. cerevisiae* cells were transformed with plasmids as indicated below and grown in liquid culture. To assay for colony formation at 25°C or 37°C, aliquots from serial dilutions of each culture, containing the number of cells indicated, were prepared and spotted onto SD-leucine plates with or without thiamine, which were incubated for 5 days. The colony formation assays were replicated independently at least three times. The results shown are representative of each of the replicates. (A) Wild-type *S. cerevisiae* cells were transformed with YEplac181 as a negative control (-ve) or with YEplac181 recombinants that contained expression cassettes in which the *S. pombe pik1* coding region, as the cDNA sequence, was under the control of the wild-type *S. pombe nmt*1 promoter sequence (P_{nmt1}-*pik1*) or by an attenuated (P_{nmt41}-*pik1*) or highly attenuated (P_{nmt81}-*pik1*) version of that promoter. (B) Wild-type *S. cerevisiae* cells were transformed with YEplac181 as a negative control (-ve) or with YEplac181 recombinants that contained a recombinant sequence encoding an eGFP-Pik1p fusion (P_{nmt41}-eGFP-*pik1*), or two allelic mutants of *pik1* encoding the eGFP-Pik1^{D709A} fusion (P_{nmt41}-eGFP-*pik1*^{D709A}) or the eGFP-Pik1^{R8383A} fusion (P_{nmt41}-eGFP-*pik1*^{R838A}) under the control of P_{nmt41}.


A
25°C
+Th
-Th
10^5 10^4 10^3 10^2 10^1 10^5 10^4 10^3 10^2 10^1
-ve
P nmt1-pik1
P nmt41-pik1
P nmt81-pik1
37°C
+Th
-Th
10^5 10^4 10^3 10^2 10^1 10^5 10^4 10^3 10^2 10^1
-ve
P nmt1-pik1
P nmt41-pik1
P nmt81-pik1
B
25°C
+Th
-Th
10^5 10^4 10^3 10^2 10^1 10^5 10^4 10^3 10^2 10^1
-ve
P nmt41-eGFP- pik1
P nmt41-eGFP- pik1R838A
P nmt41-eGFP- pik1D709A
37°C
+Th
-Th
10^5 10^4 10^3 10^2 10^1 10^5 10^4 10^3 10^2 10^1
-ve
P nmt41-eGFP- pik1
P nmt41-eGFP- pik1R838A
P nmt41-eGFP- pik1D709A

3.2. Is *S. pombe pik1* an essential gene?

The *S. cerevisiae PIK1* gene is essential for cell viability (Flanagan *et al.*, 1993). The *D. melanogaster* orthologue *fwd* is a non-essential gene; flies that carry a deletion allele of the entire *fwd* coding region are viable (Brill *et al.*, 2000). In the case of *S. cerevisiae PIK1*, its functions are not redundant to those of the two other PtdIns 4-kinases. It was not known whether *S. pombe pik1* is an essential gene. Two other putative PtdIns 4-kinases also exist in *S. pombe* (Wood *et al.*, 2002). Determination of this question is important, because it will direct future research. This section attempts to answer if *S. pombe pik1* is an essential gene by using two methods; (1) gene deletion in diploid cells, and (2) gene deletion in haploid cells carrying an episomal *pik1* copy and then loss of the episome from this particular haploid cell.

3.2.1. *S. pombe* genomic *pik1* deletion in diploid cells

3.2.1.1. Construction of a diploid *S. pombe* strain hemizygous for *pik1*

As described in section 2.1.10. (page 65), the coding region of one of the *pik1* loci in diploid cells was replaced with a *ura4* gene expression cassette by homologous recombination, resulting in the generation of a hemizygous strain N1231. A diploid strain (h^+/h^- *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*) homozygous wild-type for *pik1* (denoted as *pik1/pik1*) was transformed to uracil prototrophy by homologous recombination with the linear recombinant DNA illustrated in Figure 3.5. The genotype of strain N1231 is: h^+/h^- *pik1/pik1*::*ura4 ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18* (denoted as *pik1/*Δ*pik1*::*ura4*). Three independent clones were isolated on the selective medium, EMM lacking adenine and uracil. Deletion of the *pik1* locus was confirmed by diagnostic colony PCR (Figure 3.5. B). For this PCR, two primer sets were synthesized to confirm that the genomic *pik1* locus was replaced with the *ura4* expression cassette (Figure 3.5. A). Two primers, H1430 and H1436, were complementary to sequences 5′ and 3′ to the *pik1* non-coding region, respectively. The other two primers, H1536 and H1537, were complementary to sequences in the middle of the *ura4* expression cassette. If the gene replacement was correct in terms of location and direction, PCR products of 1.6 kb and 1.8 kb would be produced. As shown in Figure 3.5. B, all three clones contained the integrated *ura4*

expression cassette. For all following experiments, one clone has been used: N1231 *pik1* / Δ*pik1*::*ura4*.

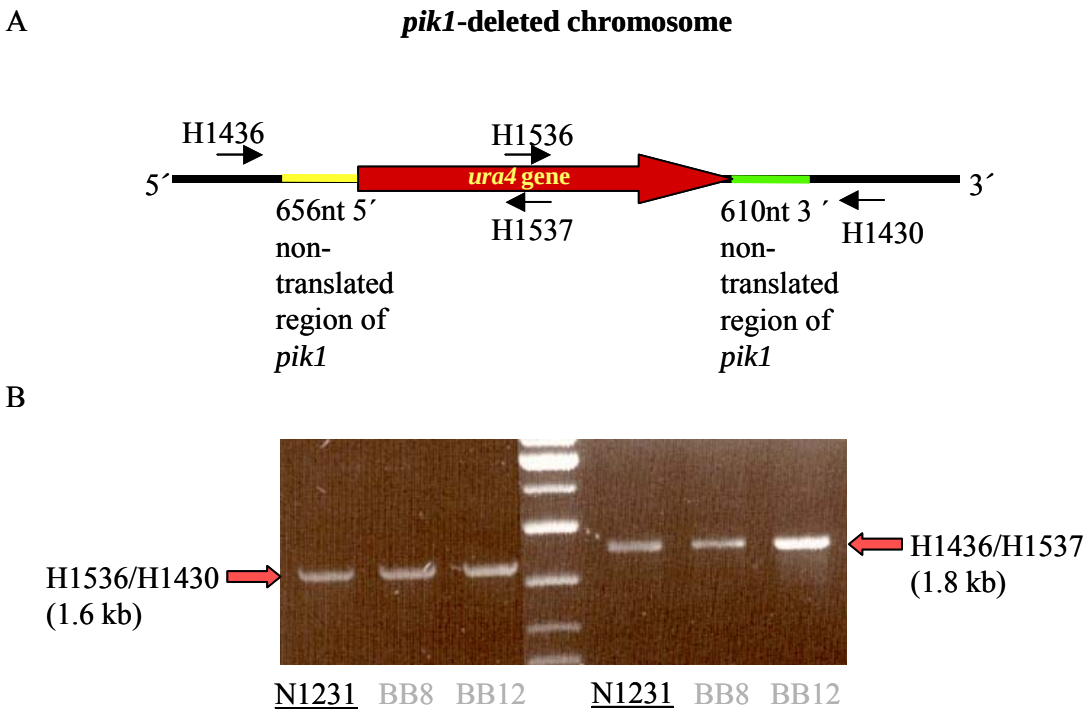


Figure 3.5. Construction of a diploid *S. pombe* strain hemizygous for *pik1*. The genomic *pik*1coding region was replaced with the *ura4* selectable marker by homologous recombination in diploid cells to generate a hemizygous diploid cell, strain N1231. The hemizygous strain N1231 was generated as described in Materials and Methods. (A) Schematic diagram of the chromosomal *pik1* locus with the coding region replaced by *ura4* cassette. Positions of primers for diagnostic PCR are indicated. To confirm that the coding region of one of the two *pik1* loci was replaced with the *ura4* gene, diagnostic PCR was performed. Primer sequences were located as shown in the figure. (B) The primer pair H1536/H1430 should amplify a product of 1.6kb. The primer pair H1436/H1537 should amplify a product of 1.8kb. Three independent clones were confirmed: N1231, BB8, and BB12. One clone, N1231, was chosen for the further work.

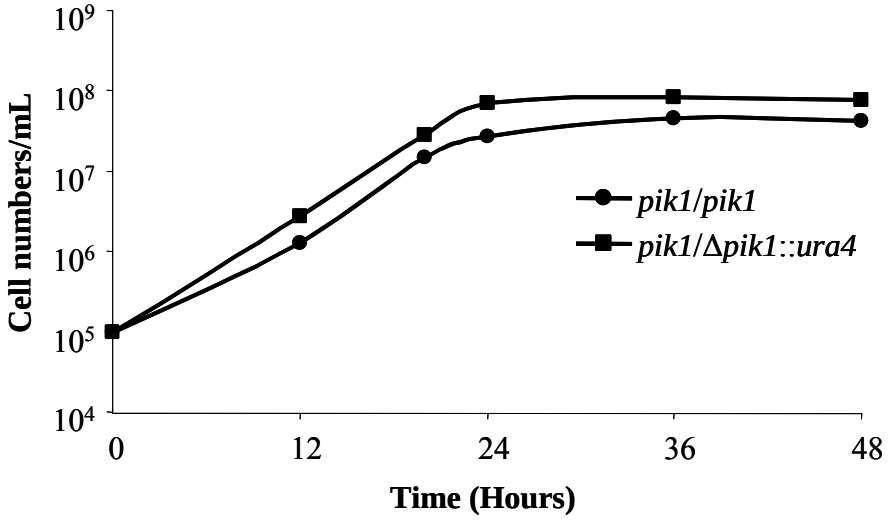
3.2.1.2. Characterization of diploid *S. pombe* strain N1231 (*pik1* / Δ*pik1*::*ura4*)

Cells of the hemizygous *pik1/*Δ*pik1*::*ura4* diploid strain were examined for cell proliferation, F-actin ring index and morphology, myosin ring index and morphology, septation index and morphology, microtubule (MT) morphology, Cdc4p subcellular distribution, and Pik1p subcellular distribution. The control cells were from the wild-type *pik1/pik1* strain. The cell proliferation curves showed no significant difference between *pik1/*Δ*pik1*::*ura4* and *pik1/pik1* cells (Figure 3.6. A, page 103). F-actin or myosin ring index is the portion of cells in which contractile rings were visualized using reagents specific for F-actin or Myo2p, respectively. The septation index is the portion of cells that have a septum as visualized by fluorescence microscopy after staining with calcofluor white or by bright-field microscopy. The contractile ring indices (F-actin ring index and myosin ring index) and septation index of the *pik1/*Δ*pik1*::*ura4* cells were similar to those of the wild-type cells (Figure 3.6. B). These results indicate that one chromosomal *pik1* gene is sufficient for cell division in diploid cells. The morphologies of the F-actin ring and septum in *pik1/*Δ*pik1*::*ura4* cells were similar to those of wild-type cells (Figure 3.7. A and B, respectively, page 104). In cells of both genotypes, the F-actin ring and septum were assembled between completely segregated nuclei. The MT organization in *pik1/*Δ*pik1*::*ura4* cells was also similar to that of wild-type cells (Figure 3.7. C, page 105); long MTs were assembled in mononuclear interphase cells of both genotypes (Figure 3.7. C, cell identified by letter 'i'), and mitotic MTs were formed as an italic *H*-shaped array or a ring structure in binuclear mitotic cells of both genotypes (Figure 3.7. C, cell identified by letter 'm'). Myo2p rings were assembled in *pik1/*Δ*pik1*::*ura4* cells like in wild-type cells (Figure 3.8. A, page 107). The myosin ring was positioned between two segregated nuclei. The hemizygous cell also displayed a wild-type Cdc4p ring between two nuclei like in wild-type cells (Figure 3.8. B). These observations indicate that a single genomic *pik1* copy is sufficient for the hemizygous diploid cell division with a normal contractile actomyosin ring (CAR) and septum formation.

S. pombe Pik1p distribution was observed in wild-type or hemizygous diploid cells by indirect immunofluoresence microscopy. A polyclonal antiserum against the C-terminal domain of *S. pombe* Pik1p was generated previously (Desautels *et al.*, 2001).

For the negative control, pre-immunized rabbit serum was also collected. Diploid cells that were wild-type or hemizygous for *pik1* were cultured until the mid-logarithmic phase of growth, and then fixed by a methanol fixation method. Half of each preparation of fixed cells was used for staining with the polyclonal anti-Pik1p serum and the other half with pre-immune serum. No significant signals were detected in wild-type or *pik1/*Δ*pik1*::*ura4* cells when the pre-immune serum was used (Figure 3.9., page 108). However, a punctate signal throughout the cytoplasm has been observed in diploid cells of both genotypes although the intensity seemed to be slightly different (Figure 3.9.). The signal in *pik1/*Δ*pik1*::*ura4* cells may represent only half of Pik1p. Nevertheless, this observation is consistent to the observation of *S. cerevisiae* Pik1p distribution; it was also distributed as a punctate dot pattern throughout cytoplasm (Sciorra *et al.*, 2005). The further investigation of the *S. pombe* Pik1p subcellular localization will be demonstrated in a section 3.4.

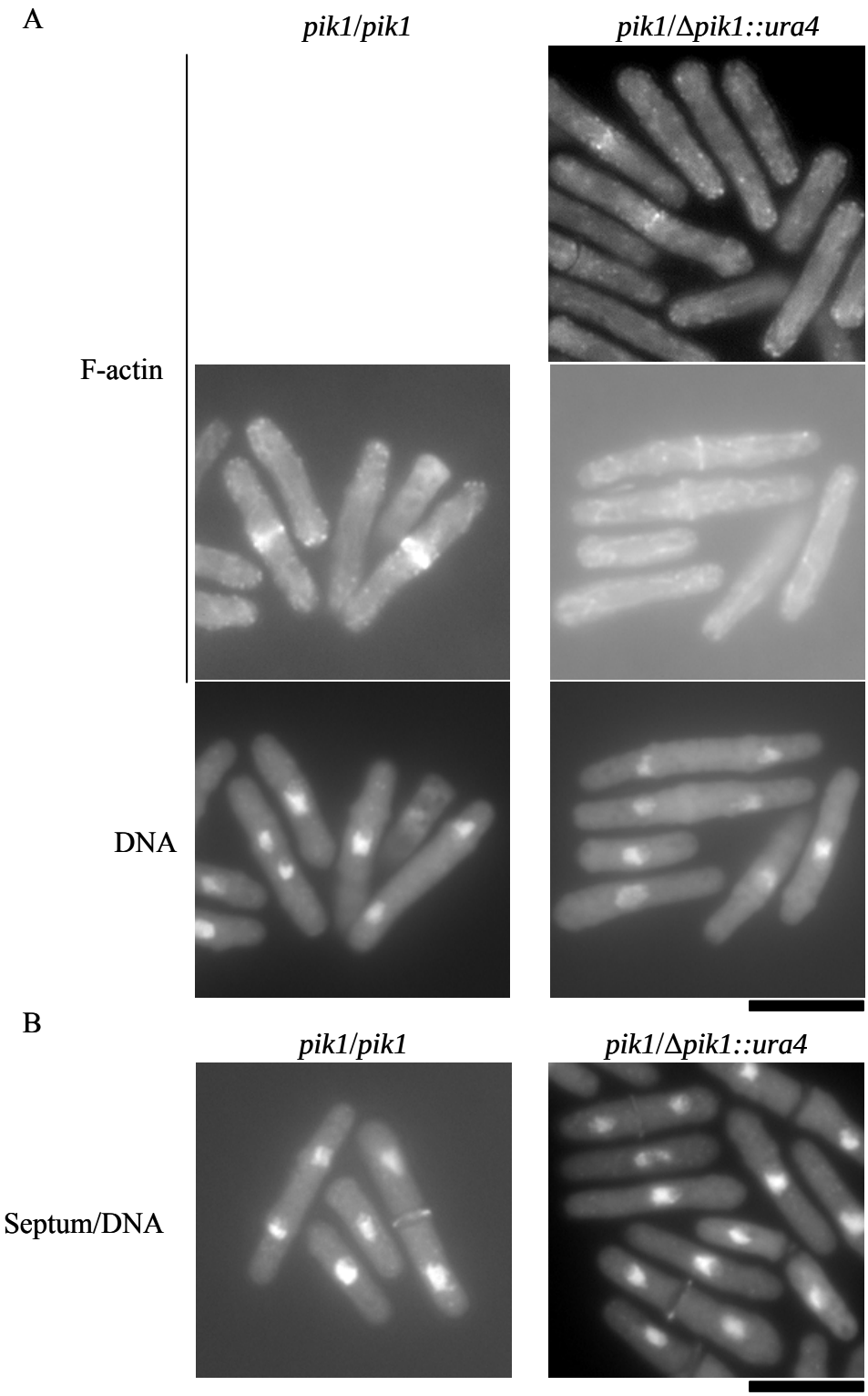
A



B

	pik1/*pik1*	*pik1*/Δ*pik1*::*ura4*
F-actin ring Index, %	11 (48/439)	8 (66/712)
Myo2p ring Index, %	13 (57/441)	15 (88/600)
Septation Index, %	11 (68/646)	9 (75/912)

Figure 3.6. Proliferation of diploid *S. pombe* cells hemizygous for *pik1*. (A) Cultures were started in 50 mL of EMM with appropriate supplements with an initial cell density of 1 X 10^5 cells/mL after preculture overnight at 30°C, and were incubated for up to 48 hours. The proliferation of wild-type diploid cells (●, *pik1*/*pik1*) and hemizygous diploid cells (N1231, ■, *pik1*/Δ*pik*1::*ura4*) was similar. (B) Cells were fixed with formaldehyde or methanol after culture for 24 hours. The indices are the proportion of the cells that possessed an F-actin ring, a Myo2p ring or a septum. These indices showed no significant difference between wild-type and hemizygous diploid cells. Numbers in brackets are the number of cells with F-actin ring, Myo2p ring, or septa that were counted.


A
pik1/pik1
pik1/Δpik1::ura4
F-actin
DNA
B
pik1/pik1
pik1/Δpik1::ura4
Septum/DNA

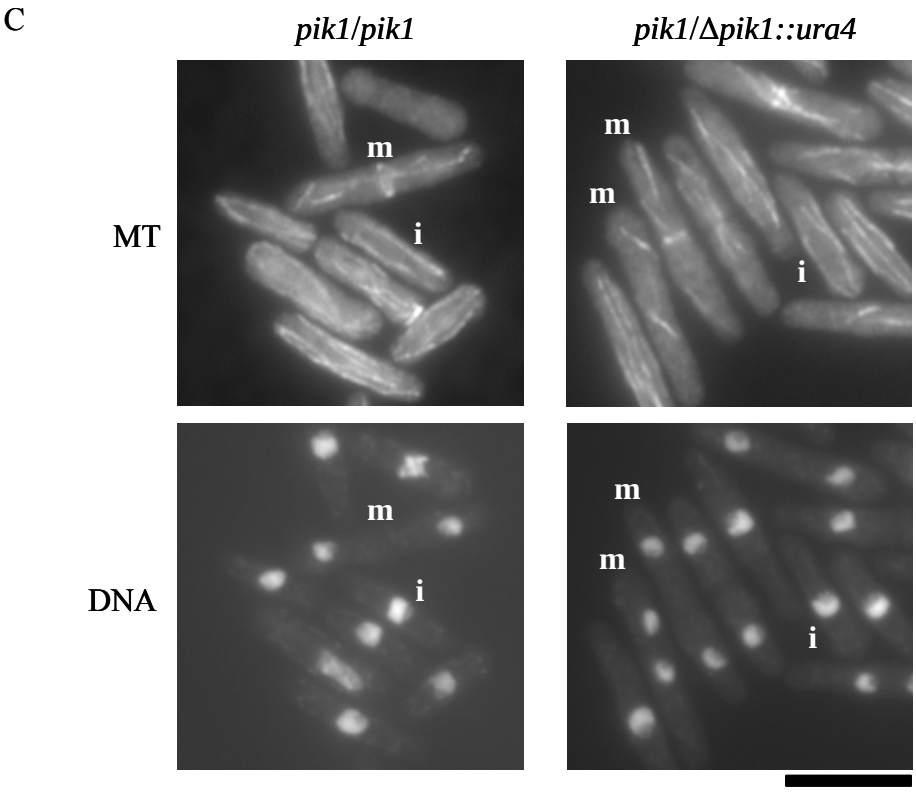
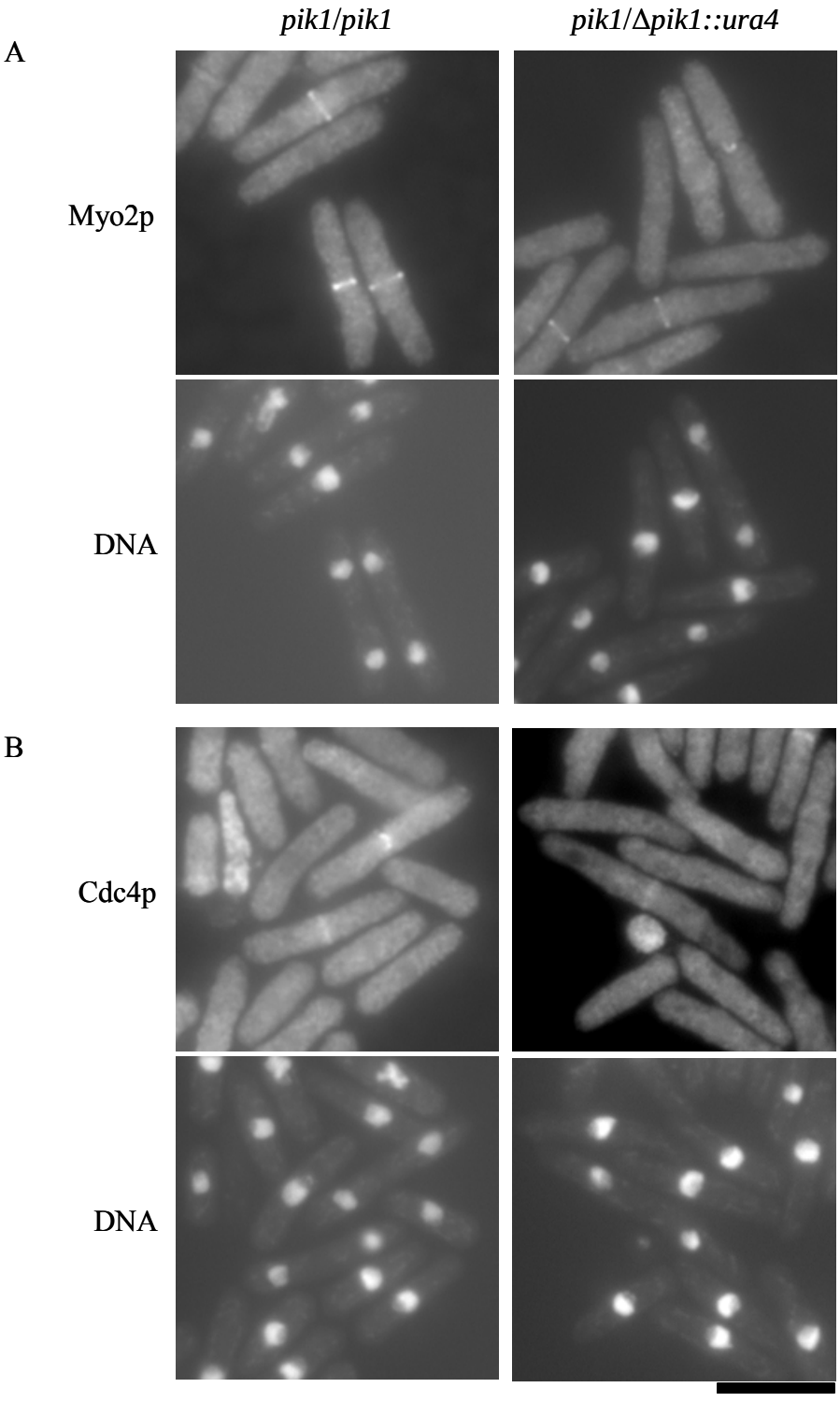


Figure 3.7. F-actin, septum, and microtubules in diploid *S. pombe* cells hemizygous for *pik1*. Cell cultures and fixation were carried out as described in Figure 3.6. (B). (A) Visualization of F-actin and nuclei in the same cells by epifluorescence microscopy after staining with FITC-conjugated phalloidin and DAPI, respectively. (B) Visualization of septum and nuclei in the same cells by epifluorescence microscopy after staining with calcofluor white and DAPI, respectively. (C) Visualization of microtubules (MTs) and nuclei in the same cells by epifluorescence microscopy after staining with antibody TAT-1 (anti-tubulin) and DAPI, respectively. The distribution or morphology of F-actin, septum, or MTs had no significant difference in wild-type and *pik1*/Δ*pik*1::*ura4* cells. m = mitotic cell, i = interphase cell, Scale bars, 10 μm.

Figure 3.8. Contractile actomyosin ring morphology in diploid *S. pombe* cells hemizygous for *pik1*. Cell cultures were carried out as described in Figure 3.6. (B). Cells were fixed with methanol. (A) Visualization of Myo2p and nuclei in the same cells by epifluorescence microscopy after staining with anti-Myo2p and DAPI, respectively. (B) Visualization of Cdc4p and nuclei in the same cells by epifluorescence microscopy after staining with anti-Cdc4p and DAPI, respectively. The medial contractile ring composed of Myo2p or Cdc4p was assembled in wild-type and hemizygous diploid cells. Scale bar, 10 μm.


pik1/pik1
pik1/Δpik1::ura4
A
Myo2p
DNA
B
Cdc4p
DNA

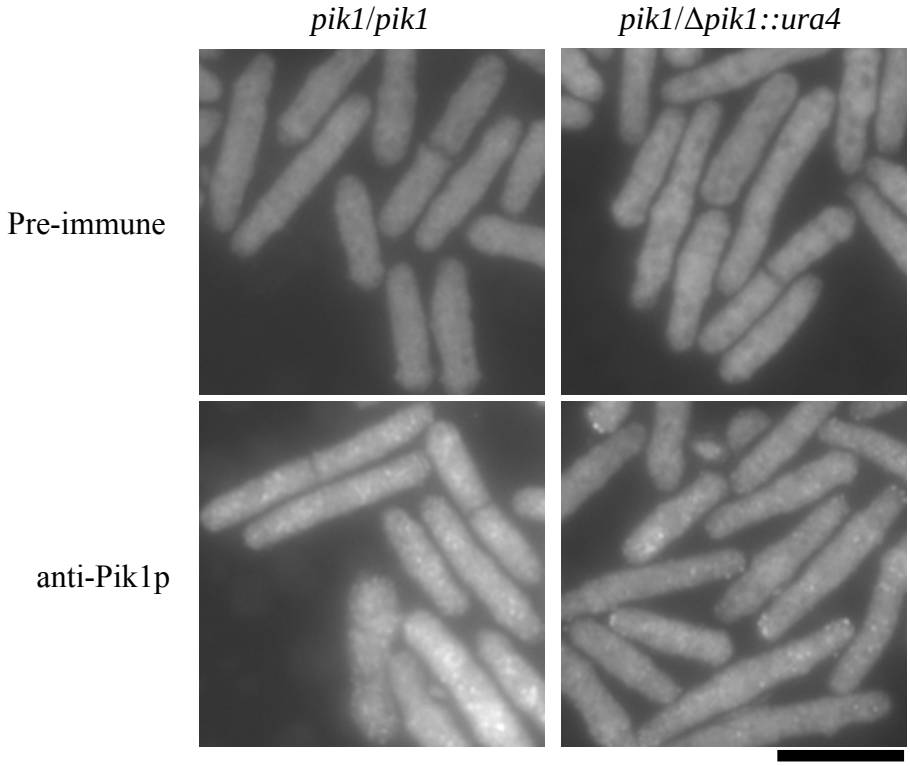


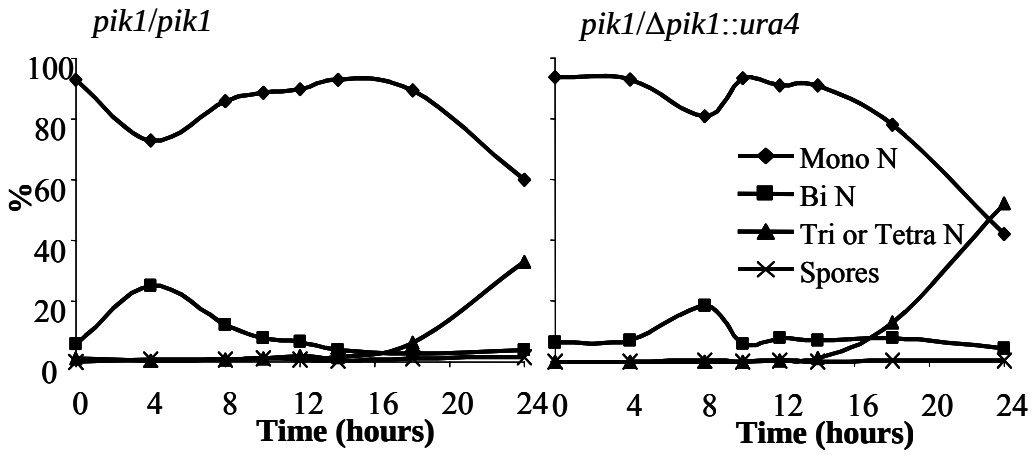
Figure 3.9. Visualization of Pik1p in diploid *S. pombe* cells hemizygous for *pik1*. Cell cultures were carried out as described in Figure 3.6. (B). Cells were fixed with methanol. Indirect immunofluorescence microscopy after treatment with a rabbit antiserum raised against part of Pik1p was carried out. Scale bar, 10 μm.

3.2.1.3. Meiosis and tetrad analysis in *S. pombe* strain N1231 (*pik1* / Δ*pik1*::*ura4*)

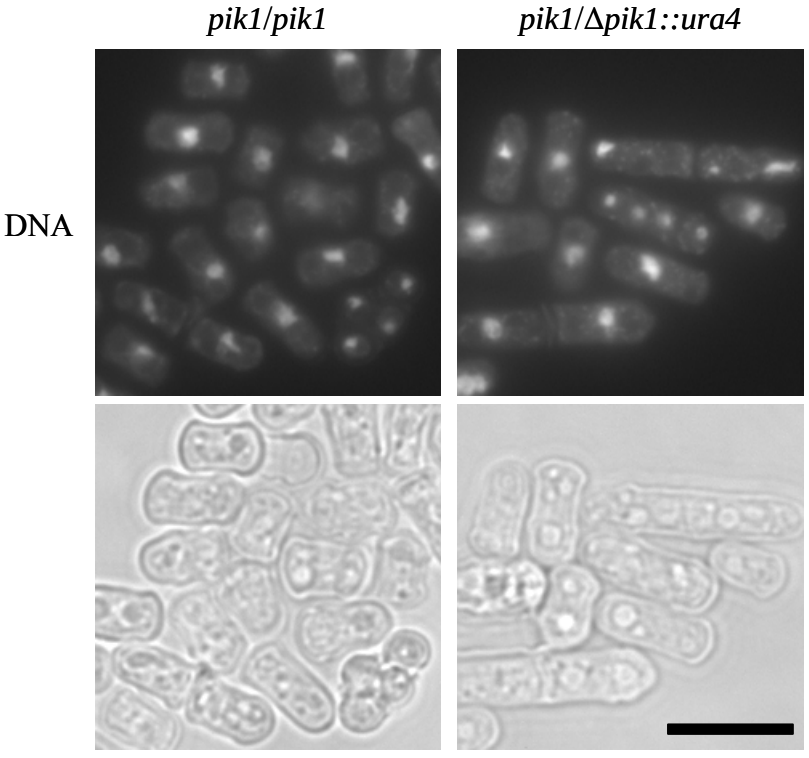
Tetrad analysis of diploid yeast cells that are hemizygous for a gene of interest can reveal whether that gene is essential. Of the four spores produced by meiosis and sporulation, two will contain the gene of interest, and two will not. If the gene of interest is essential, only two colonies will be observed. On the other hand, if the gene of interest is non-essential, all four spores will form colonies. For this analysis, the hemizygous diploid cells, however, have to be able to undergo meiosis and sporulation in the presence of only one copy of the gene of interest. I therefore monitored these processes in strain N1231 *pik1*/Δ*pik1*::*ura4* which is a uracil prototroph and in a control wild-type diploid strain *pik1/pik1* which is a uracil auxotroph. Diploid cells were precultured in rich medium (YES) at 30°C for 24 hours. After washing cells in sterile distilled water, they were placed on a medium that promotes sporulation (SPA plates) at 25°C (0 hour). Cells were collected and fixed at the indicated time points, and the fixed cells were stained with DAPI to visualize DNA. The numbers of cells that were mono-, bi-, or tri-/tetra-nucleate, and the numbers of spores were counted for each sample. The time-course observation of the meiosis of *pik1*/Δ*pik1*::*ura4* cells was similar to that of the wild-type cells except that the peak of bi-nucleated cells was delayed (Figure 3.10. A). It nevertheless indicates that meiosis in *pik1*/Δ*pik1*::*ura4* cells occurs with almost normal rate like in wild-type cells. DNA staining and bright-field microscopy results indicate correct meiosis and spore formation (Figure 3.10. B). These results suggest that a single genomic *pik1* copy is sufficient for sporulation of the hemizygous *pik1*/Δ*pik1*::*ura4* diploid cells.

Figure 3.10. Time course of meiosis and sporulation in diploid *S. pombe* cells hemizygous for *pik1*. Diploid *pik1*/*pik1* or *pik1*/Δ*pik1*::*ura4* cells were cultured overnight in EMM lacking adenine or EMM lacking adenine and uracil, respectively. Cells were washed three times with distilled water and counted. Aliquots containing $2x10^7$ cells were spotted onto an SPA medium plate at time = 0 hour. Cells were collected for formaldehyde fixation and DAPI staining at the indicated time points. (A) The timing of the peak frequency of *pik1*/Δ*pik*1::*ura4* cells with 2 nuclei appeared to have been delayed compared to that for *pik1*/*pik1* cells. The timing for the frequency of cells with 3 or 4 nucei was similar for both strains. (B) The segregating nuclei were visualized by DAPI staining after 15-hour incubation on the SPA medium plate. Asci were observed under a bright-field microscope. Scale bar, 10 μm.

A


pik1/pik1
pik1/Δpik1::ura4
100
80
60
40
20
0
%
0 4 8 12 16 20 24
Time (hours)
0 4 8 12 16 20 24
Time (hours)
Mono N
Bi N
Tri or Tetra N
Spores

B


pik1/pik1
pik1/Δpik1::ura4
DNA

3.2.2. *pik1* is required for colony formation and may be for spore germination

Tetrad analysis of the germination and growth potential of spores from the azygotic asci produced by the hemizygous diploid confirmed the essential nature of *pik1* gene expression at 30ºC. As expected, all 4 spores produced by the *pik1*/*pik1* cells germinated and formed colonies at 30ºC (Figure 3.11. left panel). Of the 4 spores from *pik1*/Δ*pik1*::*ura4* diploid cells, only two spores formed colonies (Figure 3.11. middle panel). The cells that formed colonies were wild-type for the *pik1* gene as determined by colony PCR analysis and they were able to grow on rich medium (YES) but not on minimal medium (EMM lacking uracil) (Figure 3.12.). To observe the terminal phenotypes of the spores that failed to form colonies, microscopic analysis was performed. Of the spores that did not form colonies, five remained spherical, ten displayed outgrowth, and one went division at 30ºC (Figure 3.11. right panel).

Spores from azygotic asci produced by *pik1*/Δ*pik1*::*ura4* diploid cells were incubated at different temperatures to determine whether *pik1* is essential at a range of normal growth temperatures. At 19°C, 25°C, and 36°C, only two of four spores germinated and grew to form colonies (Figure 3.13.). The cells that formed colonies were wild-type because the cells were unable to grow in medium lacking uracil, indicating that they did not carry the Δ*pik1*::*ura4* allele (Figure 3.13.). The spores that failed to form colonies at 19°C or 25°C mostly germinated and underwent up to 2 or 3 division cycles. At 36°C, the spores that did not form colonies failed to germinate, or germinated but did not undergo a cell division (Figure 3.13.). These results indicate that *S. pombe pik1* is essential for the vegetative cell division cycle at the temperatures tested as well as probably for spore germination at the highest temperature tested.

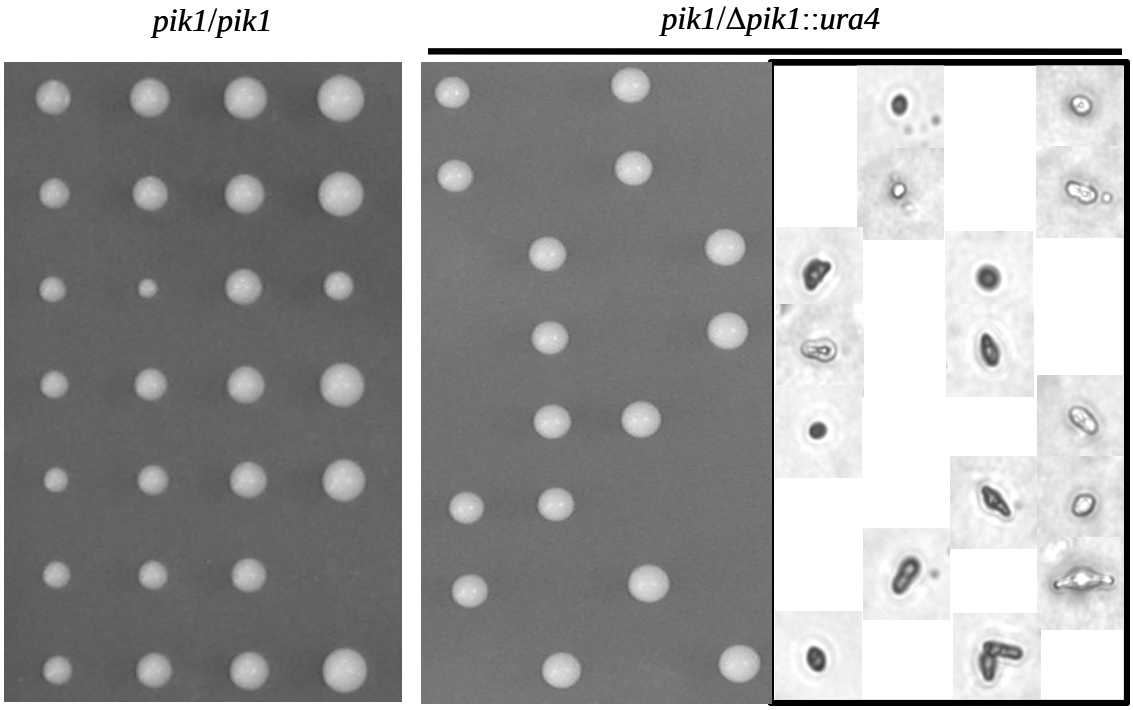


Figure 3.11. *S. pombe pik1* is an essential gene. Diploid cells *pik1/ pik1* or *pik1/ Δpik1::ura4* were incubated on ME plates at 25°C for 2 days to obtain azyogotic asci. Asci produced from the diploid cells were dissected and spores were incubated on YES plates at 30°C for 5 days. Each of four spores formed a colony when both chromosomal *pik1* loci were intact (left panel). When only one intact chromosomal *pik1* locus was present (middle panel) only 2 of the 4 spores formed colonies. In cases where colonies did not form the site of spore deposition was studied microscopically and photographed (right panel). Many spores failed to germinate. Some spores germinated, but cell division ceased after 1-2 cycles.

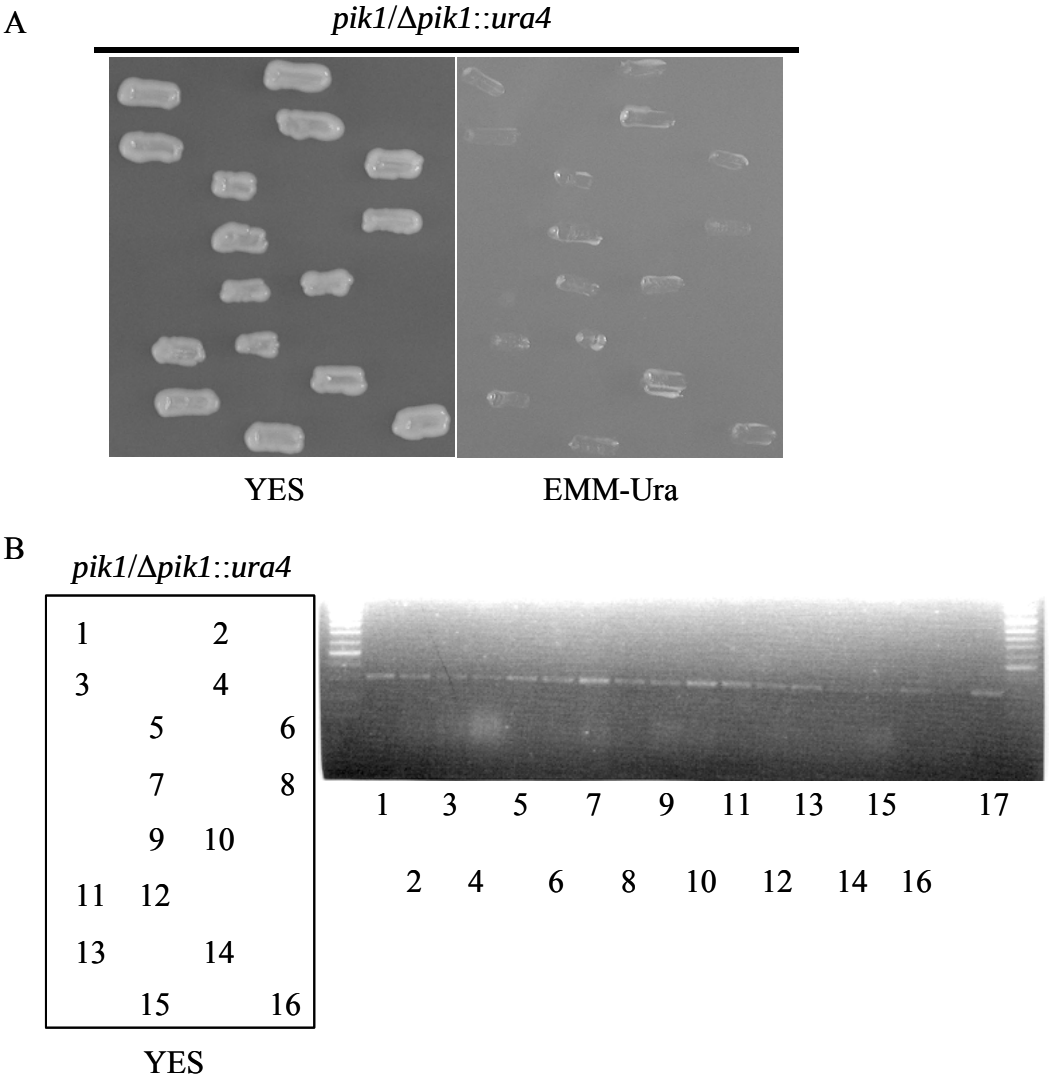
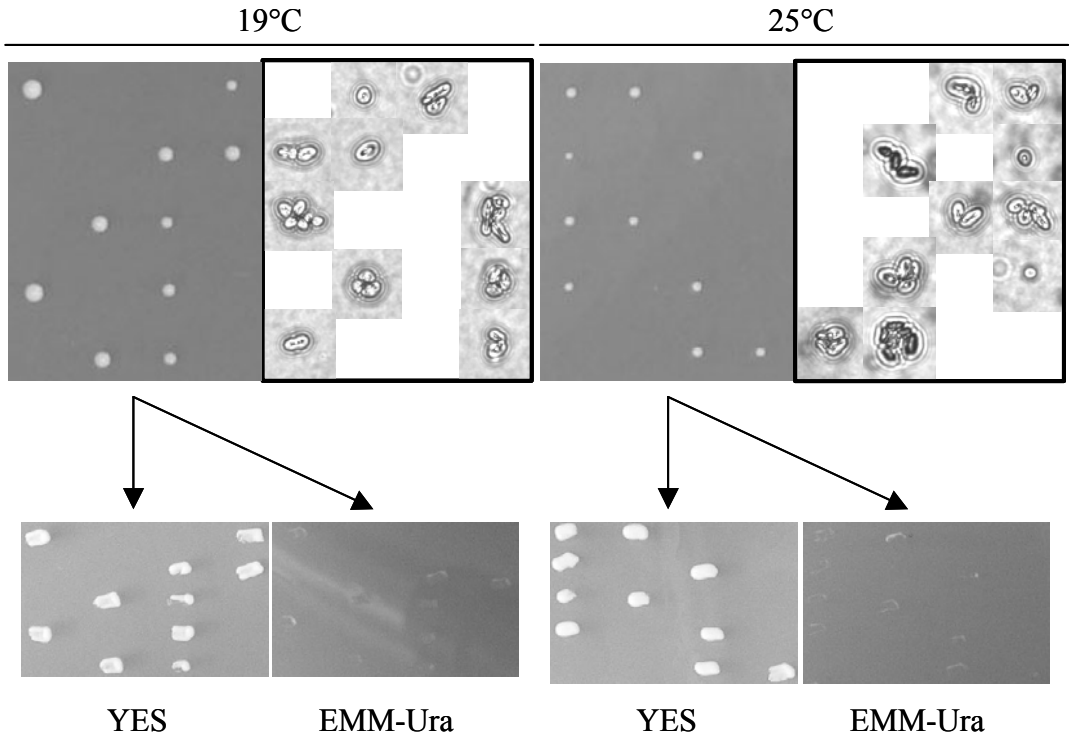
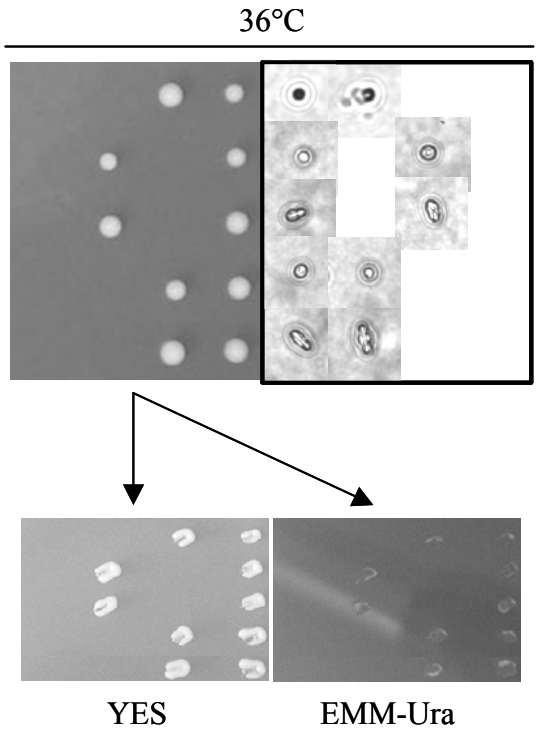


Figure 3.12. Determination of genotypes of colonies formed from spores from *pik1*/Δ*pik1*::*ura4* asci. (A) To determine the uracil requirement for the colonies shown in Figure 3.10. middle panel, those colonies were restreaked on YES plates or EMM plates lacking uracil, and incubated until growth was visible at 30°C. Colonies formed in all cases on YES, but not on EMM-uracil, indication that all colonies carried the wild-type *pik1* allele rather than the Δ*pik*1::*ura4* allele. (B) To determine if the chromosomal *pik1* locus was intact, colony PCR was performed and the PCR products were compared to the product from *pik1* haploid cells by gel electrophoresis. Lane 17 contains the product from *pik1* haploid cells.

Figure 3.13. *S. pombe pik1* is an essential gene at 19°C, 25°C and 36°C. Diploid *pik1/*Δ*pik1*::*ura4* cells were incubated on ME plates at 25°C for 2 days to obtain azygotic asci. Asci produced from the diploid cells were dissected and spores were incubated on YES plates at 25°C or 36°C for 5 days or at 19°C for 13 days. At each temperature tested colonies formed from only 2 of the 4 spores. In cases where colonies did not form, the site of spore deposition was studied microscopically and photographed. Some spores germinated, but cell division ceased after 1-2 cycles at the lower temperatures. Many spores failed to germinate at 36°C. The colonies from each temperature were restreaked on YES plates or EMM plates lacking uracil. This confirmed that the colonies required uracil, which indicates that they carried the *pik1* allele.


19°C
25°C
YES
EMM-Ura
YES
EMM-Ura


36°C
YES
EMM-Ura

3.2.3. *S. pombe* genomic *pik1* deletion in haploid cells containing an episome, pREP81-*pik1*

This approach was carried out prior to the study of gene deletion in diploid cells (described in section 3.2.1.). Wild-type haploid cells were transformed with the series of plasmid expression cassettes described in Figure 3.1. (Steinbach *et al.* unpublished). One transformant containing the episome pREP81-*pik1* (strain N1095) was chosen, because the pREP81 vector offered the lowest level of gene expression. I replaced the chromosomal *pik1* coding region with a kan^R gene expression cassette in haploid cells that carried an episomal *pik1* cDNA sequence under the control of a thiamine-repressible *nmt1* promoter to produce strain N1113. The episome, pREP81, has a highly attenuated *nmt1* promoter and a *LEU2* gene cassette for selection for leucine prototrophy (Forsburg, 1993; Maundrell, 1993). Strain N1113 cells were able to grow in rich medium containing G418 and minimal medium lacking leucine. To confirm the insertion of the kan^R expression cassette, a diagnostic PCR was carried out and the PCR products were produced from only strain N1113 DNA extract (Figure 3.14.).

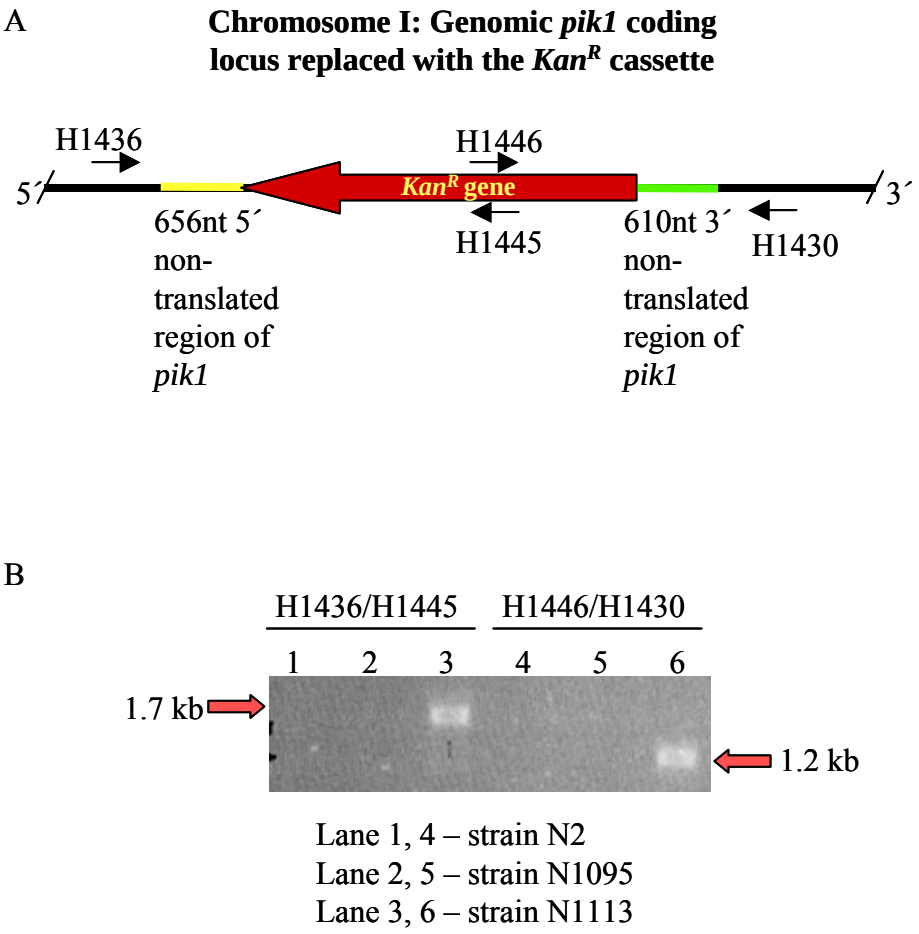


Figure 3.14. Confirmation of disruption of the genomic *pik1* locus in haploid cells by diagnostic colony PCR. The haploid strain N1113 was generated as described in Materials and Methods. (A) Schematic diagram for designing PCR primer sets. To confirm replacement of the genomic *pik1* coding region by the *Kan*^{R} cassette, PCR was performed using two sets of primers; H1436/H1445 and H1446/H1430. The primer sites are indicated in the figure. (B) Three strains were used for diagnostic colony PCR: N2, N1095, and N1113 (genotypes described in Table 2.1.). Only strain N1113 produced the diagnostic 1.7kp and 1.2kp PCR products.

I characterized strain N1113 and a control strain (N1095) that retained the intact chromosomal *pik1* locus and carried the episome pREP81-*pik1* with respect to cell proliferation, cell length, septum morphology and septation index, the distribution of F-actin or Myo2p, F-actin ring index, and Pik1p subcellular distribution. The level of *pik1* expression would be contributed by two sources: the presence or absence of the intact chromosomal *pik1* gene, and the presence or absence of thiamine to regulate the expression of the episomal *pik1* cDNA sequence. The chromosomal *pik1* gene is regulated under its own promoter. The episomal *pik1* sequence would be expressed at derespressed levels from the pREP81 vector by culturing cells in the absence of thiamine and at 'leaky' levels in the presence of the repressor, thiamine. *pik1* expression from both sources would contribute to the total Pik1p level in strain N1095, whereas the expression from the episome would be the only Pik1p source in strain N1113. Cell division control could be differently affected by the alteration of the *pik1* expression in these strains.

The cell proliferation rate was determined in the presence or absence of thiamine. Three independent experiments were performed. In all cases, cultures were started at a cell density of 1 x 10^5 cells/mL (time 0 hour) from an overnight preculture at 30°C in the presence of thiamine. Cells were counted at certain time points. The average at each time point was plotted (Figure 3.15. A, page 122). Cells of both genotypes (strains N1095 and N1113) proliferated in the presence or absence of thiamine. These cells reached saturation with similar cell numbers, approximately 10^8 cells/mL in the presence or absence of thiamine (Figure 3.15. A). However, both strains N1095 and N1113 took longer to reach to stationary cell numbers in the absence of thiamine than in the presence of thiamine (Figure 3.15. A).

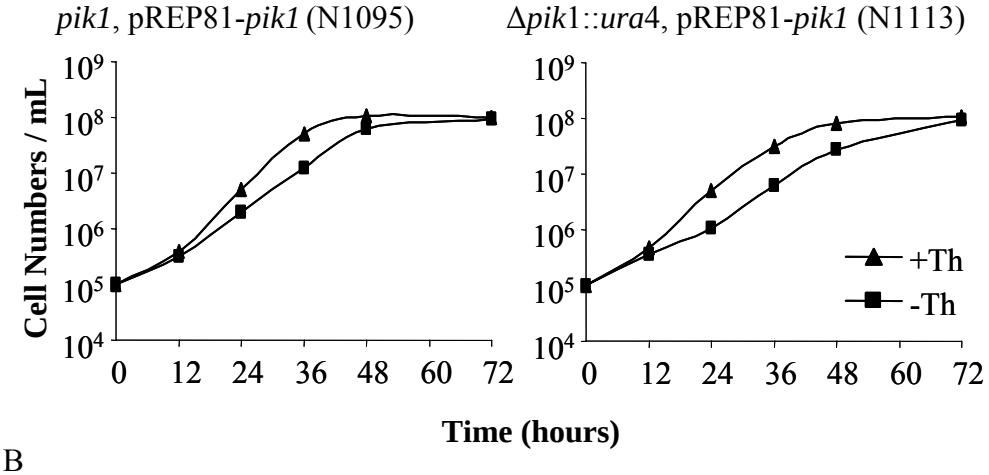
The cell length distributions in asynchronous cultures of each strain were similar regardless of the presence or absence of thiamine (Figure 3.15. B). Wild-type haploid *S. pombe* cells divide when they are in the range of 12 – 15 μm in length. Very few N1095 cells grown under derepressed condition were longer than 15 μm (Figure 3.15. C). In contrast, 8.5% of N1113 cells grown under derepressed condition were longer than 15 μm (Figure 3.15. C). The F-actin ring formation index and septation index were determined for each culture in the mid-logarithmic phase of growth. These indices were

very similar in each case (Figure 3.16. A, page 123). Septum was formed in most cells (Figure 3.16. B). A small fraction of N1113 cells in the derepressed condition were elongated and contained more than one septum (Figure 3.16. C, page 124). This phenotype was observed in 2 of 4 independent experiments. The distributions of F-actin and Myo2p were very similar in each culture (Figure 3. 16. D and E, respectively, page 125). As F-actin structures are described in section 1.1. (page 4) and depicted in Figure 1.2. (page 9), the F-actin patch was polarized at one or both tips, and F-actin rings were observed in some cells, as were Myo2p rings. The distribution of Pik1p was also determined by indirect immunofluorescence microcscopy using a polyclonal antiserum that was developed against the C-terminal 149 amino acids of Pik1p (Figure 3.17., page 127) (Desautels *et al.*, 2001). The cytoplasmic punctate pattern was observed under most conditions except in N1113 cells in the presence of thiamine. The distribution of the punctate pattern was not observed in cells treated with preimmune serum as a negative control. The N1113 cells in the presence of thiamine however had no visible Pik1p distribution. It is worth noting that the repression of the *nmt1* promoter is leaky and some level of expression is observed, in the presence of thiamine (Forsburg, 1993). Therefore, these results indicate that in cells carrying the Δ*pik1*::*Kan*^{R} chromosomal allele, the level of *pik1* expression from the thiamine-repressed, highly attenuated *nmt1* promoter is sufficient for cell viability and cell cycle control, but insufficient for the detection of Pik1p by indirect immunofluorescence microscopy.

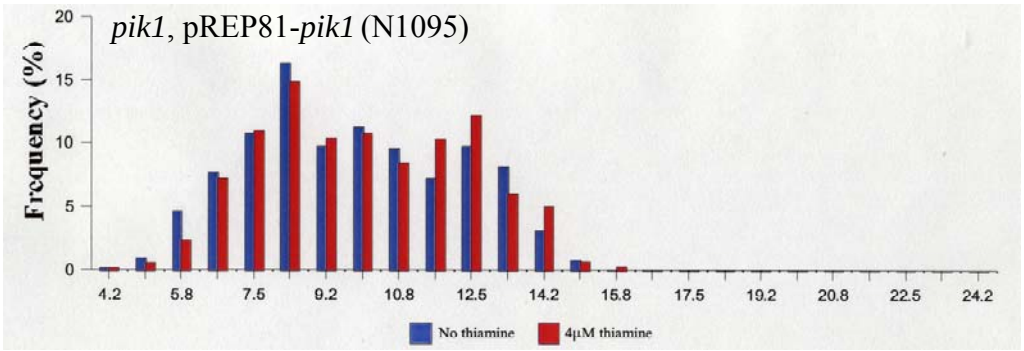
Due to the viability of N1113 cells in the repressed condition, I altered the direction of experimental design to answer the question whether *pik1* gene expression is essential for vegetative growth. I attempted to lose the episome pREP81-*pik1* in the cells lacking the chromosomal *pik1* locus. If *pik1* were non-essential, culturing these cells in the presence of leucine to relax the selection for leucine prototrophy should result in the accumulation of cells that lack the episome, and *pik1* gene function. Repeated attempts failed to identify cells that had lost the episome. This result suggests that *S. pombe pik1* is essential for the vegetative growth, which is consistent with the finding from the tetrad dissection analysis.

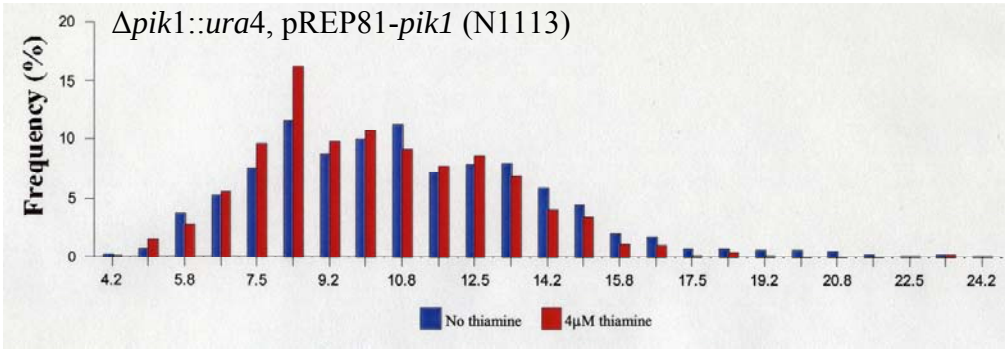
Figure 3.15. Cell proliferation and cell length distribution of strains N1095 and N1113. (A) Proliferation of cells of strains N1095 and N1113 at 30^{o}C. Cultures were started from an overnight pre-culture at 30°C in the presence of thiamine. In each case, the initial cell density was 1 x 10^5 cells/mL (time 0 hour) in the presence (▲) or absence (■) of thiamine and cells were cultured for 72 hours. These independent experiments were performed. The average cell density at each time point was plotted. In each experiment, the cells reached the same density at saturation and in each case, the cultures grown in the absence of thiamine took longer to reach stationary phase than did the cultures grown in the presence of thiamine. (B) Cell length distribution of strains N1095 and N1113. Cell cultures started at cell density of 1 x 10^5 cells/mL in the presence or absence of thiamine for 24 hours, and then cells were fixed with formaldehyde. Cell lengths were estimated relative to a micrometer bar by bright-field microscopy. Cell length distribution was similar in all cases except in cells possessing the derepressed ectopic *pik1* expression in the absence of chromosomal *pik1* locus. (C) Proportion of cells, which exceed the length 15 μm. 8.5% of the Δ*pik1*::*ura4*, pREP81-*pik1* cells in the absence of thiamine were longer than 15 μm.

A



B





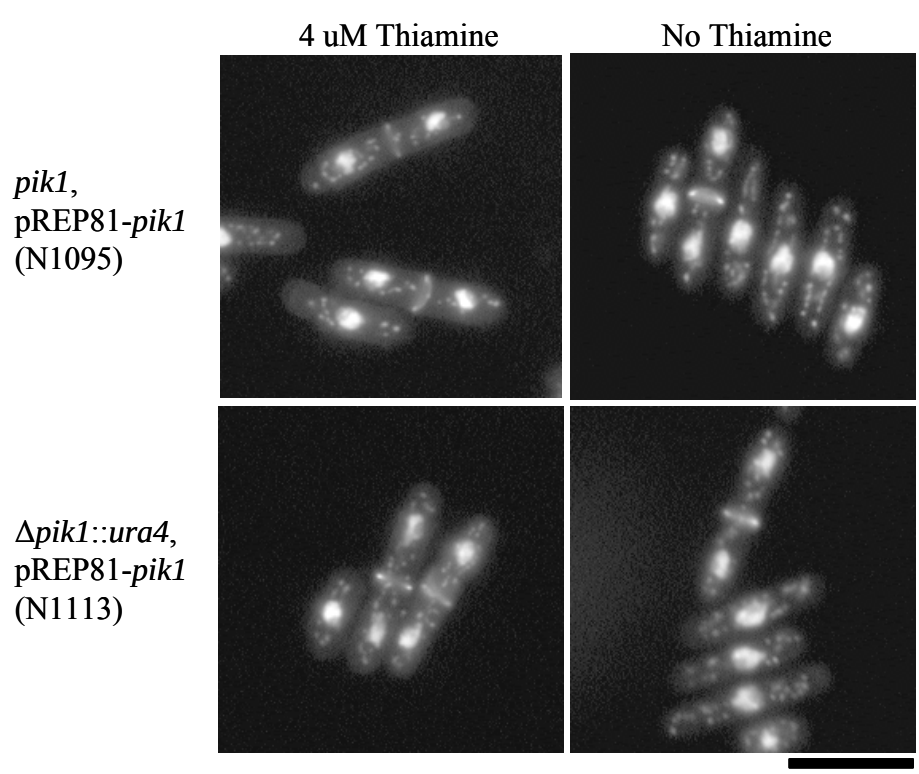
C

	Strain			
	N1095		N1113	
Thiamine	+	-	+	-
Proportion of cells longer than 15μm (%)	0.6	0.4	2.0	8.5

A

	Strain			
	N1095		N1113	
Thiamine	+	-	+	-
F-actin Ring Index (%)	8	8	9	8
Cell numbers	18/233	26/336	21/222	13/159
Septation Index (%)	8	8	12	10
Cell numbers	139/1824	76/965	209/1800	213/2045

B



C

No Thiamine

Δ*pik1*::*ura4*,
pREP81-*pik1*
(N1113)

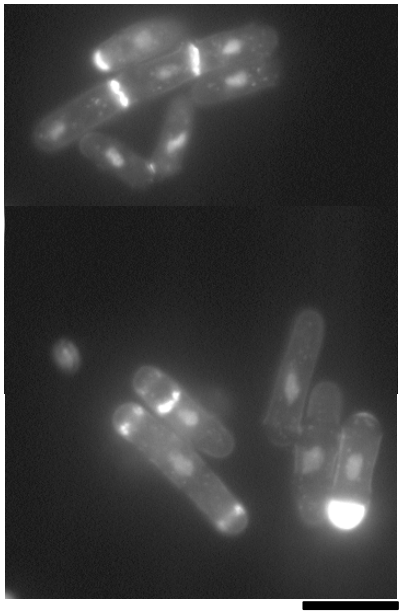
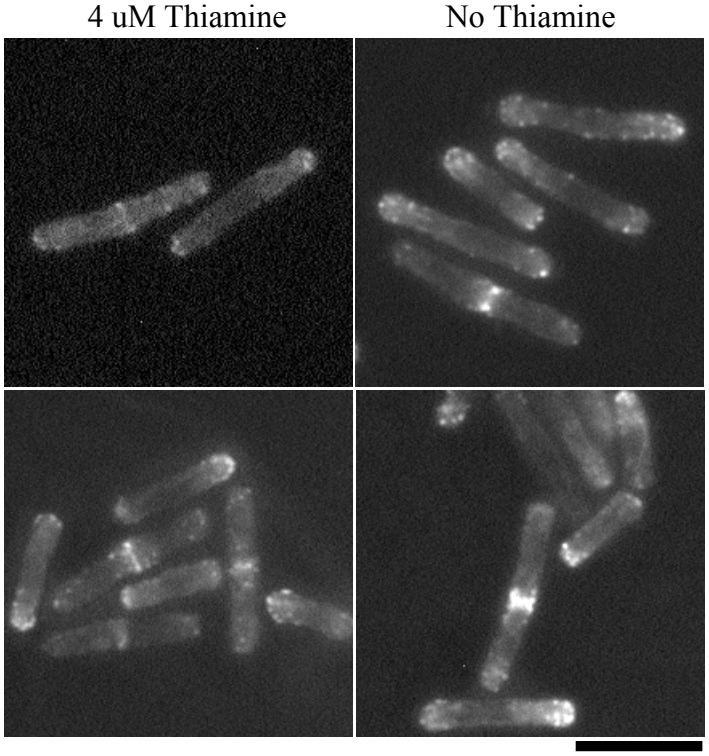


Figure 3.16. Distributions of F-actin and Myo2p, and septum morphology in strains N1095 and N1113. The strains and culture conditions used were as described in Figure 3.15 (B). Cells were fixed with formaldehyde for septum staining with calcofluor white, and for F-actin visualization with FITC-phalloidin, and for DNA staining with DAPI. Cells were fixed with methanol for Myo2p immunostaining. (A) F-actin ring and septation indices were determined. Cell numbers with F-actin ring or septum per total cell numbers counted are indicated. (B) Septum morphology and nuclear DNA were visualized and appeared to be normal. (C) Some Δ*pik*1::*ura4*, pREP81-*pik1* cells in the absence of thiamine showed elongation with multiple septa, multiple nuclei, or abnormal septum material accumulation. (D) F-actin distribution was observed. F-actin patch distribution was polarized at one or both tips, and F-actin ring was assembled in cells regardless of the presence or absence of thiamine. (E) Myo2p was visualized using indirect immunostaining with an anti-Myo2p antibody. Medial Myo2p accumulation was observed in both strains regardless of the presence or absence of thiamine. Scale bars, 10μm.

D



E

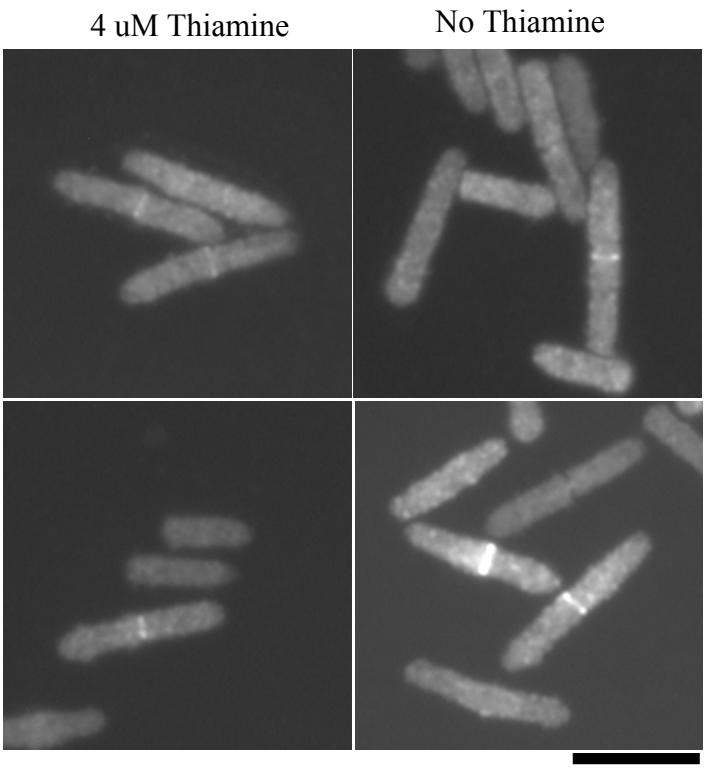
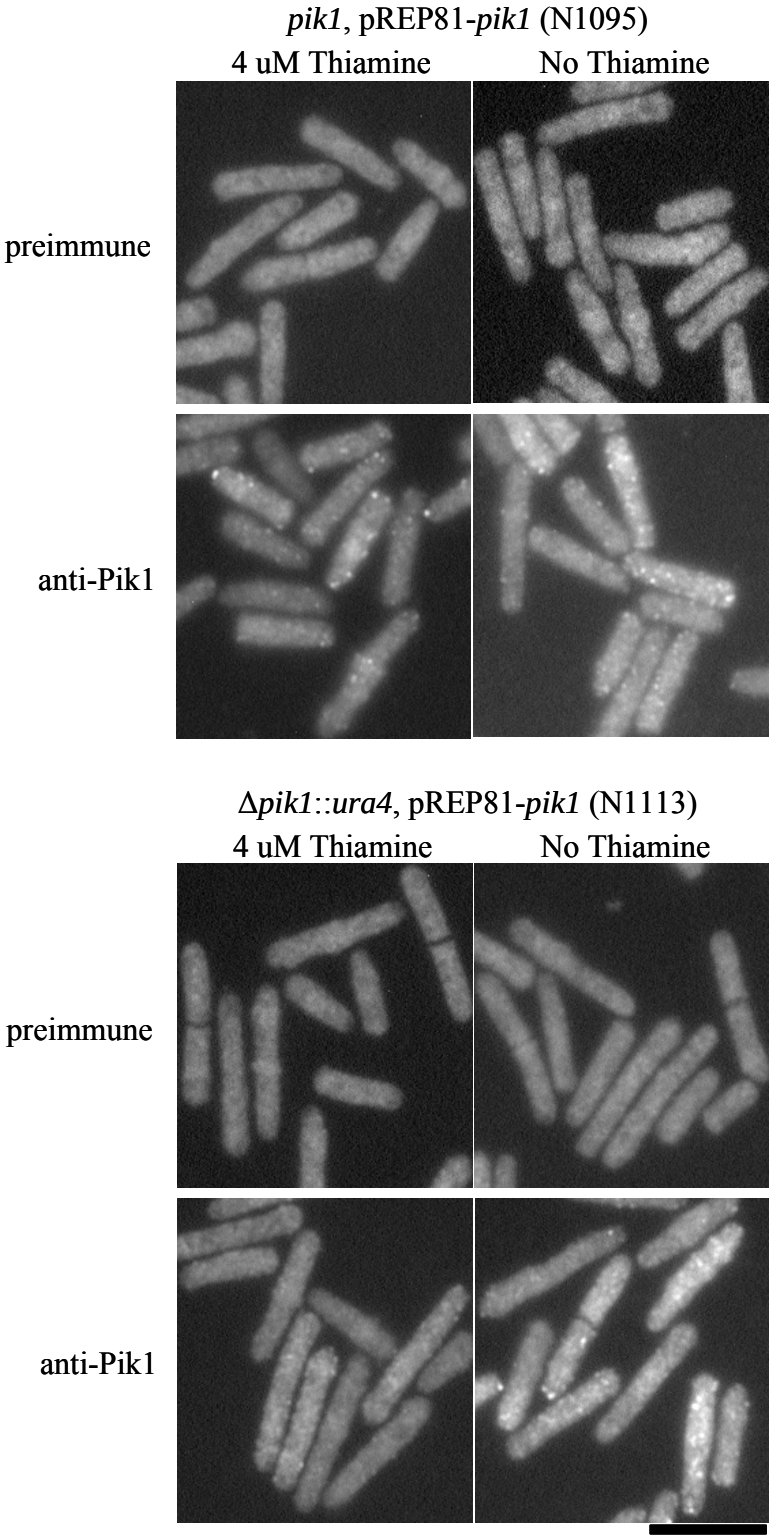


Figure 3.16. (continued)

Figure 3.17. Subcellular distribution of Pik1p in strains N1095 and N1113. Culture conditions were as described in Figure 3.15. (C). Cells were fixed with methanol for indirect immunofluorescence microscopy. A rabbit polyclonal anti-Pik1 serum or preimmune serum was used. A punctate pattern of fluorescence was observed throughout the cytoplasm in most conditions except in Δ*pik*1::*ura4*, pREP81-*pik1* cells in the presence of thiamine. In this case, the signal was weaker than for this strain in the absence of thiamine, or for strain N1095 in the presence or absence of thiamine. This observation was consistent in two trials. Scale bar, 10 μm.


pik1, pREP81-*pik1* (N1095)
4 uM Thiamine
No Thiamine
preimmune
anti-Pik1
Δ*pik1*::*ura4*, pREP81-*pik1* (N1113)
4 uM Thiamine
No Thiamine
preimmune
anti-Pik1

3.3. *S. pombe pik1* is required for cytokinesis

In the above sections, I have determined that *S. pombe pik1* is required for haploid cell division and probably for spore germination at the hightest temperature tested, evidenced by gene deletion in diploid cells and tetrad dissection analysis, and gene deletion in haploid cells carrying an episome pREP81-*pik1* and plasmid-loss. To further study the role of *pik1*, it would be advantageous to have a conditional allele; that is, an allele that would be functional under a permissive condition and would lose its function(s) under a non-permissive condition. The common conditional allele used in *S. pombe* genetics is temperature-sensitive; a conditional allele would be viable at a permissive temperature (25°C), but not at a restrictive temperature (36°C). To achieve this goal, two methods were used: site-directed mutagenesis, and fusion of the *pik1* coding sequence to a sequence that encodes a protein (N-degron) that is subjecte to temperature-dependent proteolysis.

3.3.1. *S. pombe pik*1^{A831}, homologous to an *S. cerevisiae* residue required for enzymatic activity, is not required for cell division in *S. pombe*

To determine the effect of loss-of-function of Pik1p during cell division, the first approach to generate a conditional, temperature-sensitive *S. pombe pik1* mutant was site-directed mutagenesis. The results of our complementation study suggested that *S. pombe* Pik1p confers the essential functions of *S. cerevisiae* Pik1p (Figure 3.2.). The *S. cerevisiae pik1-101* ts allele has an amino acid substitution from serine to phenylalanine at a residue 1045 (Walch-Solimena and Novick, 1999). Based on a sequence alignment of the C-terminal catalytic domains of *S. cerevisiae* and *S. pombe* Pik1p, I performed site-directed mutagenesis to substitute alanine (GCA) with phenylalanine (TTT) at residue 831 in *S. pombe pik1* cDNA (Figure 3.18.), expecting to obtain a conditional *S. pombe pik1* ts mutant. As the result of a PCR error, I also obtained a mutation, alanine (GCA) to valine (GTA) at residue 831 of the *S. pombe pik1* cDNA sequence. I introduced each mutation into *pik1* cDNA sequences in the expression vector pREP41 and transformed the hemizygous diploid *S. pombe* cells. After random sporulation, I selected haploid cells that carried the chromosomal *pik1*::*ura4*$^{+}$ allele with each resulting construct. Ectopic expression of *pik*1^{A831F} or *pik*1^{A831V} supported colony formation at

both 25°C and 36°C (data not shown). Thus, the *S. pombe pik1* A831 residue is not required for colony formation, unlike the *S. cerevisiae PIK1* S1045 residue.

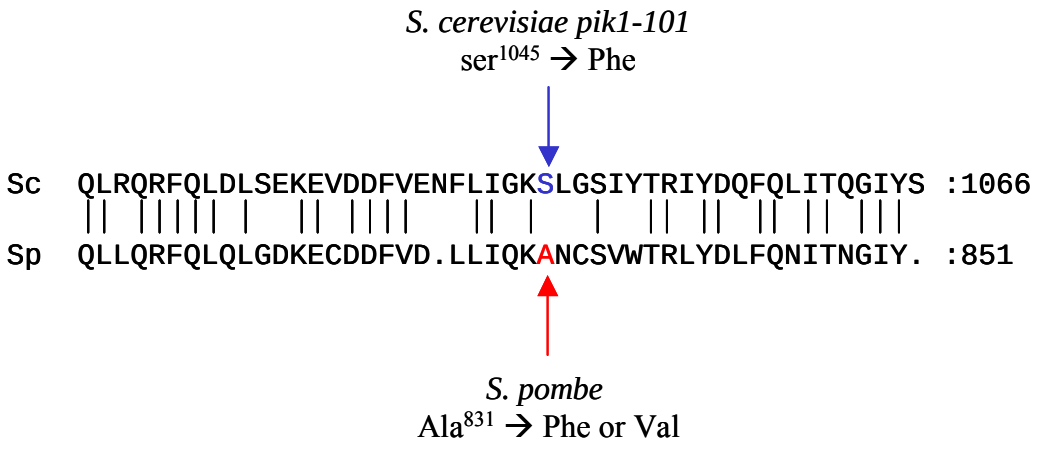


Figure 3.18. Site-directed mutagenesis of *S. pombe pik1*. The amino acid sequences of the C-terminal domains of *S. pombe pik1* and *S. cerevisiae PIK1* were aligned. The *S. cerevisiae pik1-101* ts allele carries a single mutation, Ser^{1045} to Phe (70). The corresponding residue in *S. pombe pik1*, Ala^{831}, was mutated to Phe or Val by using site-directed mutagenesis.

3.3.2. Characterization of strain N1366 *pik1-td* cells at 25°C and 36°C

3.3.2.1. Design and construction of an N-degron fusion allele of *pik1*

To assess the effects of the loss of Pik1p function, I utilized the N-degron approach. The N-degron is a conditional temperature-dependent degradation tag that was first designed for *S. cerevisiae* (Dohmen *et al.*, 1994). It is composed of a ubiquitin (Ub) coding region fused in frame to an arginine codon, and a temperature-sensitive mouse dihydrofolate reductase (Ub-R-DHFR^{ts}; N-degron) (Dohmen *et al.*, 1994). Ubiquitin is removed from the primary translation product and arginine becomes the N-terminal amino acid of a fusion protein in eukaryotic cells (Bachmair *et al.*, 1986). The initial arginine is essential for the shorter half-life of the whole fusion protein on the basis of the N-end rule (Varshavsky, 1992). The DHFR^{ts} conformation is altered upon temperature shift to 36°C, leading to the exposure of its internal lysines toward the cytosol. The exposed lysines are polyubiquitinated for ubiquitin-dependent proteolysis at 36°C. The N-degron fused protein is supposed to be functional at 25°C, but its function should be lost at 36°C *via* ubiquitin-dependent proteolysis (Figure 3. 19.). This tag is fused to the N-terminus of the protein of interest (Dohmen *et al.*, 1994). Rajagopalan *et al*. demonstrated the usage of the N-degron in *S. pombe* (Rajagopalan *et al.*, 2004). The N-degron approach is only useful for proteins tolerant to N-terminal tagging. The plasmid constructed for the N-degron approach is depicted in Figure 2.3.

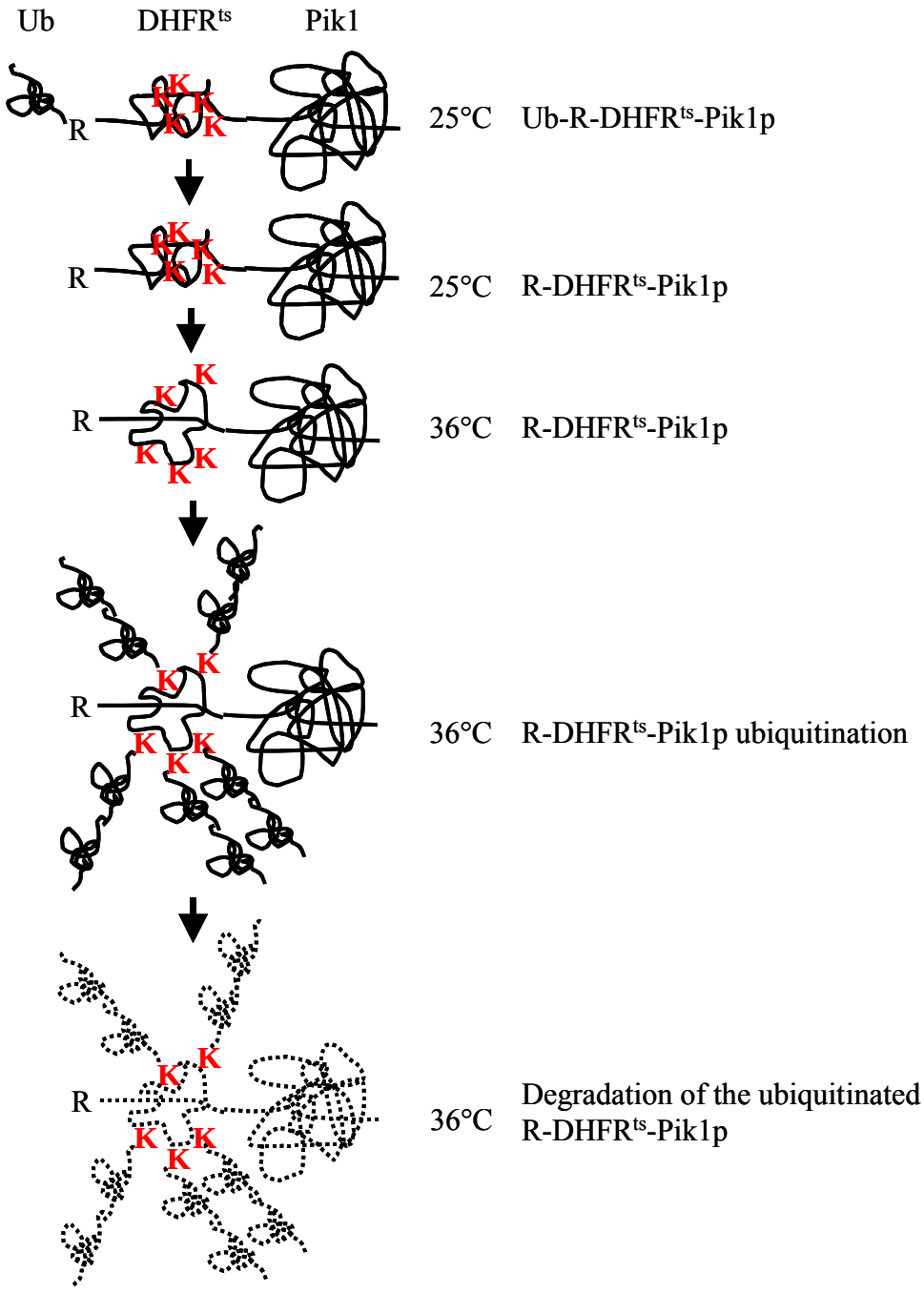


Figure 3.19. Schematic diagram of the loss-of-function of the N-degron-*pik1* fusion allele. Ub = ubiquitin, R = arginine, $DHFR^{ts}$ = mouse dihydrofolate reductase temperature-sensitive mutant, K = lysine. A solid line indicates a stable fusion protein, and a dashed line indicates a degraded fusion protein.

3.3.2.2. *S. pombe* strain N1366: Δ*pik1*::*ura4* pREP41X-Ub-R-DHFR^{ts}-*pik1*

I generated cells lacking the chromosomal *pik1* locus but containing, as the only Pik1p source, the N-degron fused episomal *pik1* coding allele (Ub-R-DHFR^{ts}-*pik1*) under the control of the attenuated *nmt1* promoter in pREP41X, referred to as *pik1-td* cells (strain N1366). I first cloned the Ub-R- DHFR^{ts}-*pik1* fusion in the leucine selectable expression vector pREP41X (pREP41X- Ub-R- DHFR^{ts}-*pik1*). This episome was introduced into hemizygous diploid *pik1*/Δ*pik1*::*ura4* cells (strain N1231; h^+/h^- *pik1*/Δ*pik1*::*ura4 ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*) by lithium acetate transformation (details in section 2.1.6.). The transformed diploid cells were selected on EMM medium lacking adenine, uracil, and leucine at 25°C. The absence of adenine from the medium selected for diploid cells since haploid cells cannot proliferate in the absence of adenine. The absence of uracil selected for the presence of the Δ*pik1*::*ura4* allele, and the absence of leucine selected for cells that contained the episome. One transformed colony was selected and used for random sporulation. The spores were plated on a selective medium plate and incubated until visible colony formation at 25°C. One colony was selected, strain N1366, which is referred to as *pik1-td* cells. As *S. pombe pik1* is an essential gene (Figure 3.11., page 113), the selection of *pik1-td* cells indicates that the N-degron fusion is sufficient at 25°C for viability of cells lacking the chromosomal *pik1* coding locus.

In the next three subsections, I characterized the *pik1-td* cells at a permissive (25°C) and at a restrictive temperatures (36°C). First, to further assess whether the N-degron fusion *pik1* allele is conditionally functional, a cell proliferation study was carried out at 25°C and 36°C.

3.3.2.3. Cell proliferation of strain N1366 is inhibited at the restrictive temperature

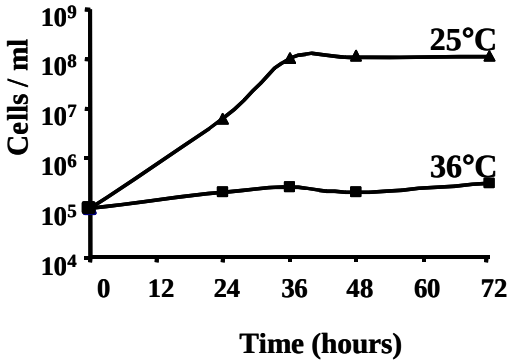
I have shown above (section 3.2.2.) that haploid cells carrying the Δ*pik1*::*ura4* allele are not viable and cannot form colonies. I have also shown, immediately above, that *pik1-td* cells are viable at 25°C as a result of the activity of the plasmid encoded N-degron – Pik1 fusion protein. If the function of the N-degron – Pik1 protein in *pik1-td* cells is lost at the restrictive temperature, then the cells should lose viability and the ability to proliferate when cultured at 36°C. The growth of *pik1-td* cells (strain N1366)

at 25°C was exponential for more than 24 hours, after which stationary phase was entered at a density of greater than 10^8 cells per mL, an increase of 3 orders of magnitude over the course of the experiment (Figure 3.20. A, page136). The doubling time was approximately 3.6 hours during the exponential phase of growth. In contrast, *pik1-td* cells (strain N1366) incubated at 36°C showed only a modest increase in cell number even after 72 hours in culture (Figure 3.20.A). Similar experiments were performed with this strain a number of times, always with similar results. At most, cell numbers increased 2 to 4 fold after shifting growth from 25°C to 36°C. I conclude from this that the N-degron – Pik1 fusion protein loses its function at 36°C, or at least that levels of function at 36°C are not sufficient to support cell proliferation.

For bright-field microscopy, two culture flasks of *pik1-td* cells were started at a cell density of 1 x 10^5 cells/mL at 25°C. One culture was incubated for 20 – 24 hours at 25°C. The other culture was incubated for 12 hours at 25°C, and then shifted to 36°C and then incubated for a further 18 hours at 36°C. Bright-field microscopy was carried out with these live cells. At 25°C, the *pik1-td* cells were cylindrical in shape and some were septating (Figure 3.20. B). However, at 36°C, most *pik1-td* cells had septa (Figure 3.20. B). In some cells, multiple septa were formed. Some daughter cells, up to 33%, seemed to be in the process of septum hydrolysis or to be arrested at the middle of septation because they were connected through some residual septum. Only 6% of cells showed this phenotpye at 25^{o}C. Also, there were cells that had proceeded through the next round of septation without completion of a previous cell separation. This phenotype was not observed at 25^{o}C (Figure 3.20. B inset). Some dumbbell-shaped cells were observed at 36°C (Figure 3.20. B). These results suggest that upon shift to 36°C the loss of function of the ectopicly expressed N-degron fusion Pik1 protein inhibits cell proliferation and seems to affect cytokinesis.

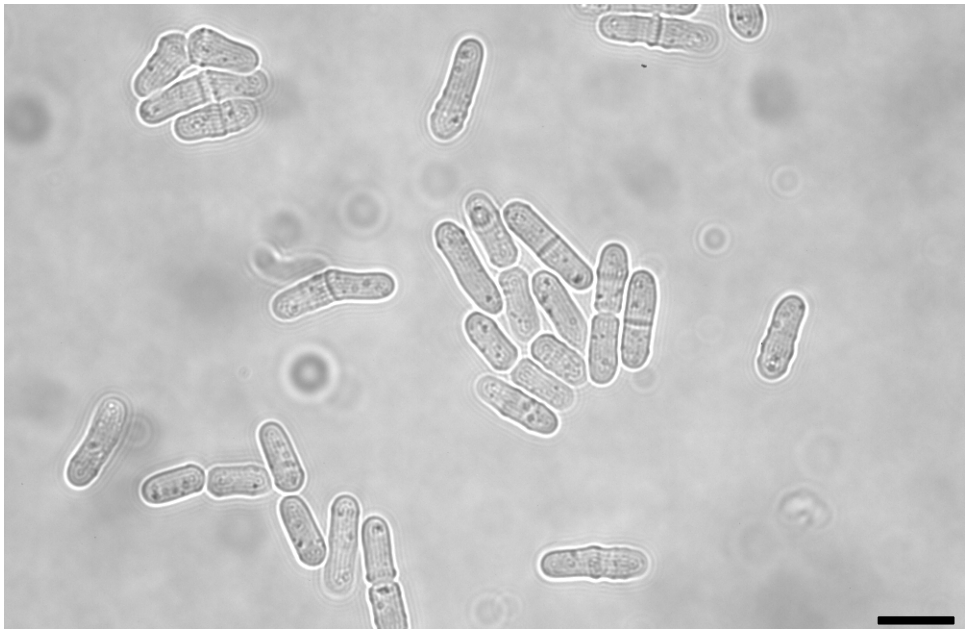
Figure 3.20. Loss of function of Pik1p causes a defect in cell division. A Ub-R-DHFR^{ts}-Pik1 fusion protein was the only source of Pik1p in *pik1-td* cells that carried a chromosomal *pik1* locus in which the coding region had been replaced with a *ura4* cassette. (A) The proliferation of *pik1-td* cells at 25°C (▲) or 36°C (■) for up to 72 hours in the presence of thiamine. Cultures were started at a cell density of 1 x 10^5 cells/mL (time 0 hour) from an overnight pre-culture at 25°C in the presence of thiamine. Cell proliferation ceased after a modest increase at 36°C. (B) Bright field microscopy of live cells. Liquid cultures of *pik1-td* cells were started at cell density of 1 x 10^5 cells/mL in the presence of thiamine. Cells in one flask were cultured for 24 hours at 25°C. Cells in a second flask were incubated at 25°C for 12 hours and then shifted to 36°C for 18 hours. Cells were collected for bright-field microscopy without fixation. In *pik1-td* cells at 36°C, cells containing multiple septa were observed or two septating cells remained attached often forming a V-shaped form (inset). Some cells at 36°C were dumbbell-shape rather than being cylindrical. These phenotypes were not observed at 25°C. Scale bars, 10 μm.

A

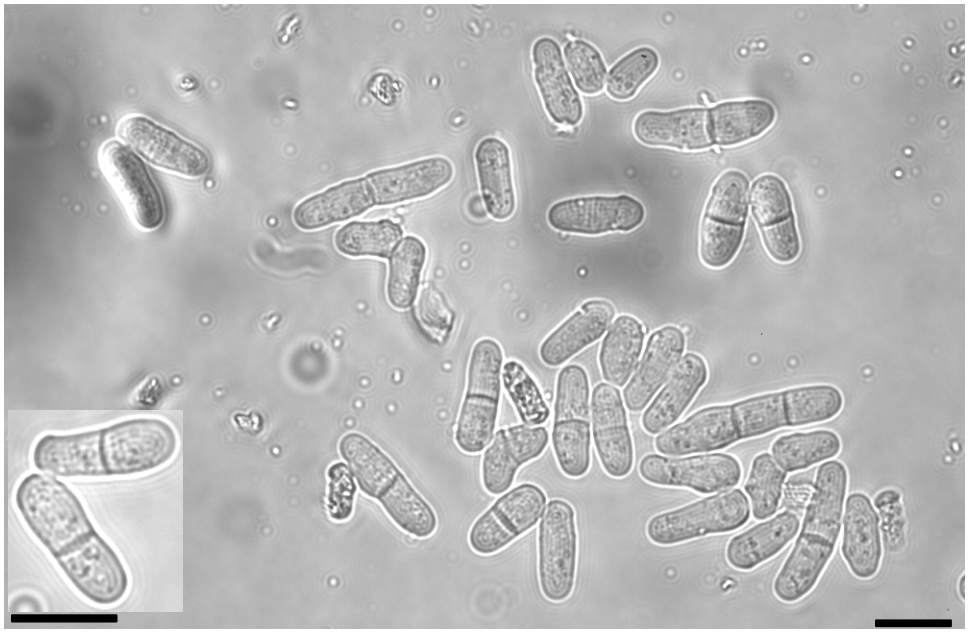

Cells / ml
10^9
10^8
10^7
10^6
10^5
10^4
25°C
36°C
0
12
24
36
48
60
72
Time (hours)

B

25°C



36°C



3.3.2.4. Actomyosin ring assembles and constricts at the restrictive temperature

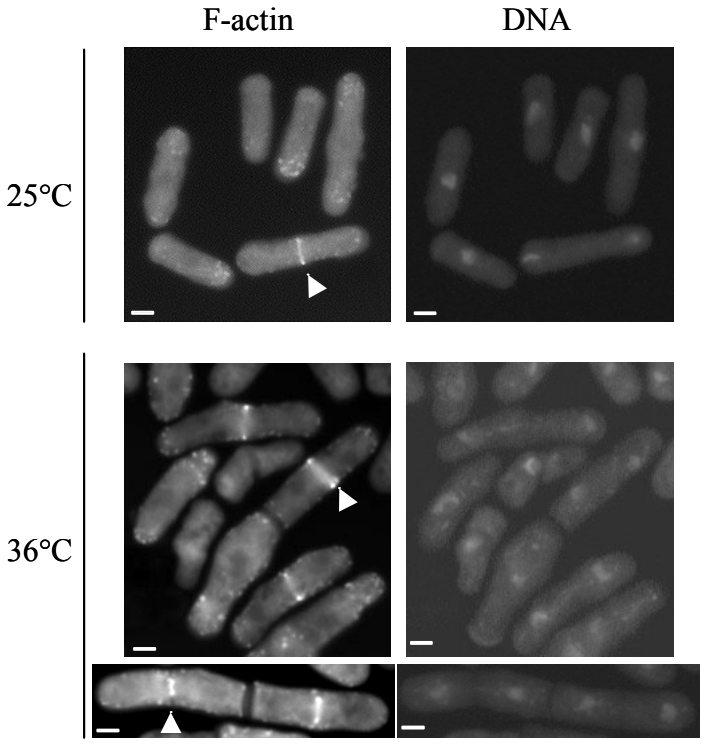
To determine what aspects of cell division were affected when *S. pombe* cells lost Pik1p functions, I examined *pik1-td* cells at 25°C or 36°C using microscopic analysis. To collect cells at the mid-logarithmic phase of growth, two cultures were started at cell density of 1 x 10^5 cells/mL. One culture of *pik1-td* cells was incubated at 25°C for 20 – 24 hours and then cells were collected. These cells were fixed by the formaldehyde method. On the other hand, the other culture of *pik1-td* cells was incubated for 12 hours at 25°C, and then shifted to 36°C for another 18 hours, resulting in loss-of-function of the N-degron fusion Pik1 protein. Cells at 36°C were collected, and fixed by the formaldehyde method (section 2.4.1.). F-actin ring indices were determined at 25°C and 36°C to be 13% and 16%, respectively (Figure 3.21. A). F-actin distribution was visualized by fluorescence microscopy after staining with FITC-conjugated phalloidin. At 25°C, F-actin was visualized at the cell tips and as a medial band (Figure 3.21. B). As was the case for cells grown at 25°C, assembled F-actin rings, and rings that appeared to have assembled and constricted were observed in cells grown at 36°C (Figure 3.21. B). In contrast to the case for cells grown at 25°C, the F-actin patch distribution was no longer polarized in cells grown at 36°C; rather they appeared to be scattered throughout the cytoplasm (Figure 3.21. B). Multiple F-actin ring assembly was observed at 36°C within multinucleated cells. The proportion of mono-, bi- or multi-nucleated cells at 25°C and 36°C were designated in Figure 3.21 (A). The proportion of cells with 2 or more nuclei was dramatically increased at 36°C. These observations indicate that *S. pombe pik1* is involved in cytokinesis rather than nuclear division. Furthermore, the observations of the F-actin distribution suggest that *S. pombe* Pik1p is required for the F-actin patch distribution during cell cycle. It appears however that Pik1p is not required for F-actin ring assembly and constriction.

Figure 3.21. F-actin rings assemble and appear to constrict in *pik1-td* cells at the restrictive temperature. Cell culture conditions were as described in Figure 3.20. (B). Collected cells were fixed with formaldehyde. To visualize F-actin or DNA, fixed cells were stained with FITC-phalloidin or DAPI, respectively. (A) At 25^{o}C, the proportion of cells with more than 1 nucleus was 17%. At 36^{o}C, 67% of cells had more than 1 nucleus. However, *pik1-td* cells had only a slightly increased F-actin ring index at 36°C compared to 25^{o}C. Numbers in brackets are cell numbers with 1, 2, 3 or more nuclei or F-actin ring. (B) F-actin ring assembly and apparent constriction took place at both temperatures (arrowheads) between completely segregated nuclei. Some cells at 36°C possessed multiple F-actin rings. F-actin patches were dispersed throughout the cytoplasm at 36°C, whereas F-actin patches were polarized at one or both tips of cell at 25°C. Scale bars, 2 μm.

A

	Proportion of cells	
	25°C	36°C
1 nucleus	84% (1687)	33% (286)
2 nuclei	17% (333)	52% (456)
≥3 nuclei	0% (0)	15% (129)
F-actin Ring Index	13% (27)	16% (129)

B



3.3.2.5. Abnormal septum morphology is observed at the restrictive temperature

As described above, most *pik1-td* cells arrested with 2 or more nuclei at 36°C. The F-actin ring however appeared to be assembled and constricted normally in *pik1-td* cells at 36°C. I thus asked whether the number of septated cells was changed upon the loss of Pik1p functions. To answer this question, septation index was determined at 25°C or 36°C. The septation index at 36°C remarkably increased up to 74%, whereas it was 13% at 25°C (Figure 3.22., page 142). Septum morphology was visualized with calcofluor white at 25°C or 36°C. Septum morphologies observed in cells grown at 36°C included; (1) intensely fluorescent septa, (2) parallel and/or multiple septa in cells with 2 or more nuclei, and (3) undissolved septum material persisting between two cells that remained unseparated, often in a V-shape (Figures 3.22.). In contrast, at 25°C a normal septum was assembled at the medial plane of cells with completely segregated nuclei (Figure 3.22.). It seems that the loss of Pik1p functions affects septation and cell separation. To further observe septum morphology, transmission electron microscopy was used to compare the wild-type and *pik1-td* cells at 25°C or 36°C. The *pik1-td* cell culture was as previously described for fluorescence microscopy and cultured at 25°C or 36°C. The wild-type cell culture at 25^{o}C was the same as the *pik1-td* cell culture. At 36^{o}C, the wild-type cells were cultured for 24 hours. The cells were fixed with potassium permanganate as described in section 2.4.8. This work was performed by P.A.Netto. The septum viewed by TEM is composed of 3 layers, one bright layer (primary septum) between two dark layers (secondary septa) (Humbel *et al.*, 2001). Septum morphology was similar in wild-type and *pik1-td* cells grown at 25°C; a fine septum with one light layer between two dark layers was observed (Figure 3.23. A, page 144). However, septum morphology in *pik1-td* cells grown at 36°C was aberrant; septa were thickened, especially the secondary septum layers, and new septa were formed in cells that still contained a previous septum (Figure 3.23. B, page 145). These observations were consistent with the results of septum visualization with Calcofluor white. Furthermore, the intracellular membranous or vacuole-like structures in *pik1-td* cells have been remarkably accumulated at 36ºC and to some extent at 25ºC (Figure 3.23. C, page 147). These structures were not observed in wild-type cells at either temperature (Figure 3.23. C).

In summary, the Ub-R-DHFR^{ts} fusion to the amino terminus of Pik1p had no apparent effect on Pik1p functions during cytokinesis at 25ºC although some intracellular membranous or vacuole-like structures were found. The loss of *S. pombe pik1* functions however perturbed the regulation of septum formation and morphology, and the dynamics of intracellular membranous and vacuole-like structures at the restrictive temperature. Thus, *S. pombe pik1* appears to be required for the completion of cell division, especially for septation and cell separation.

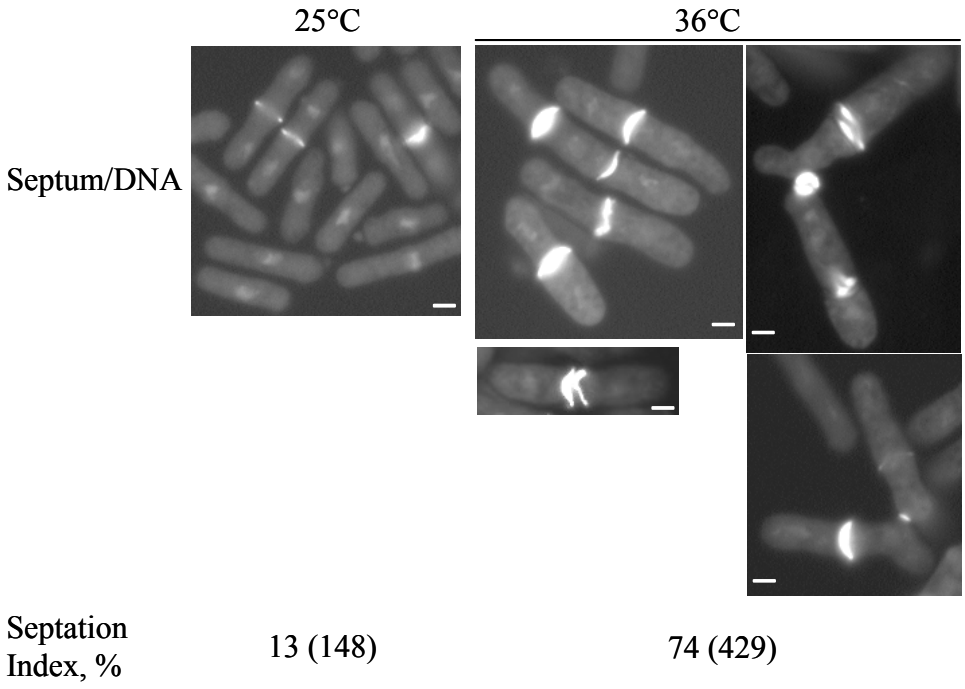


Figure 3.22. Abnormal septum formation of the *pik1-td* cells at the restrictive temperature. Cultures were performed as described in Figure 3.20. (B). Collected cells were fixed with formaldehyde. To visualize septa, fixed cells were stained with calcofluor white. Remarkably, after growth at 36°C, 74% of the cells contained one or more septa. The *pik1-td* cells cultured at 36°C had intense fluorescent signals at sites of septation. Multiple septa were observed in many cells (16 % of total cells), and daughter cells that remained attached had undergone the next round of septation. On the other hand, cells at 25°C formed fine medial septa and the septation index in these cells was 13%. Numbers in brackets are cells numbers with septa. Scale bars, 2 μm.

Figure 3.23. Temperature-dependent *pik1-td* cells are defective in septation and cell separation. Cell cultures were performed as described in Figure 3.20. (B). Cells were fixed with potassium permanganate for transmission electron microscopic analysis. (A) Wild-type or *pik1-td* cells at 25°C. A fine septum was formed at the middle of both cells. The septum was composed of a bright layer between two dark layers, shown in the magnified squares. Furthermore, vacuole-like structures were observed in *pik1-td* cells. (B) Wild-type or *pik1-td* cells at 36°C. The septum morphology was not altered upon temperature shift in the wild-type cell, forming a fine three-layered septum under transmission electron microscope. On the other hand, the septum morphology was dramatically altered upon temperature shift in the *pik1-td* cell; (i) septum was thickened especially the dark layers, (ii, iii) a second septum was formed close to a previous septum, or (iv, v) a second septum was formed apart from a previous septum. Vacuole-like and extra internal membranous structures were intensively accumulated at 36°C, whereas these structures were not observed at 25°C. (C) Non-septating cells. The vacuole-like and extra internal membranous structures observed in *pik1-td* dividing cells were also accumulated in non-septating *pik1-td* cells regardless of temperature shift although the intensity was different at 25°C and 36°C. The internal abnormality was severe at 36°C. It was shown in the magnified square. However, these structures were not observed in wild-type cells regardless of temperature shift. Scale is designated.

A

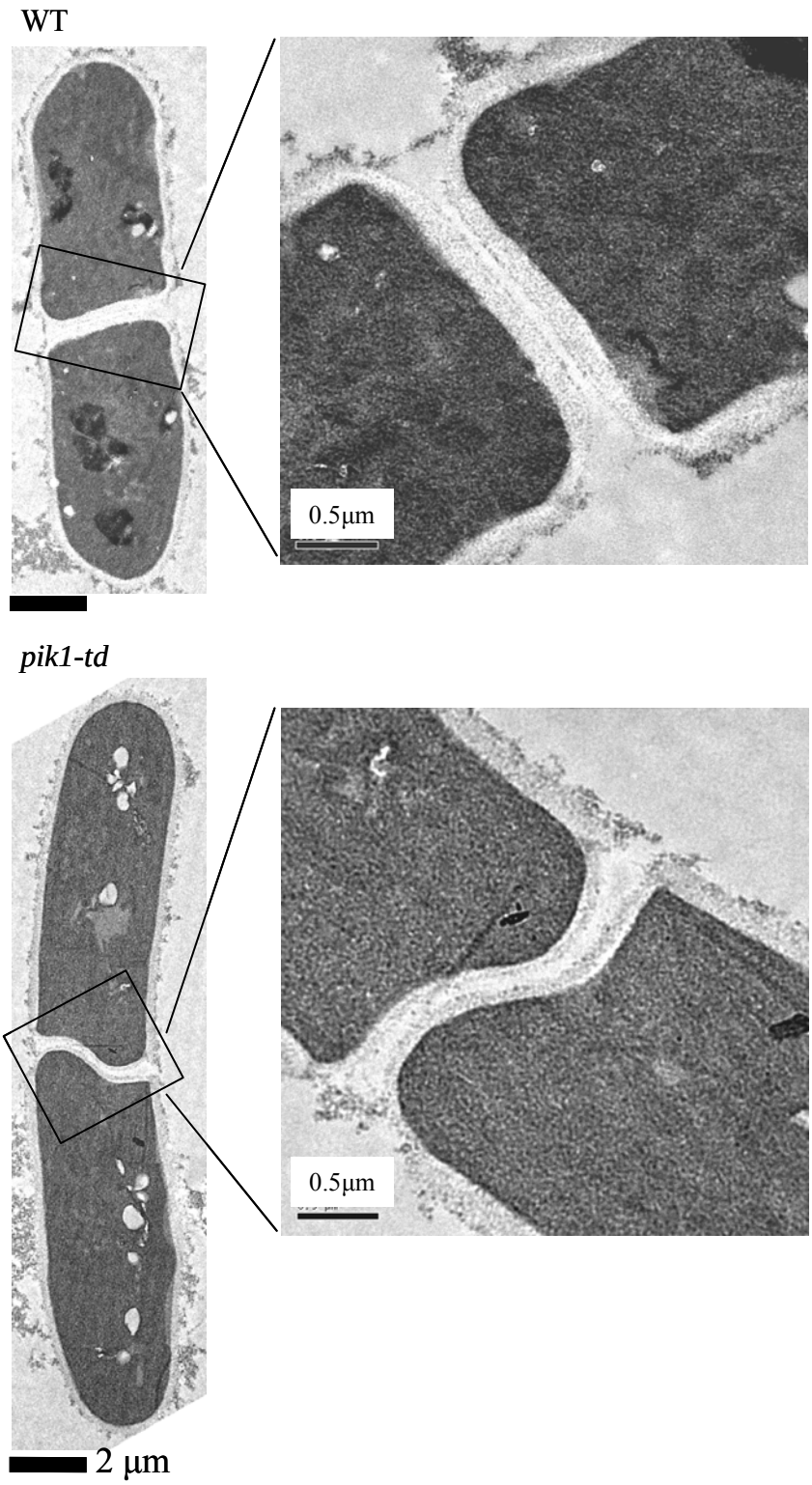


Figure 3.23.

B

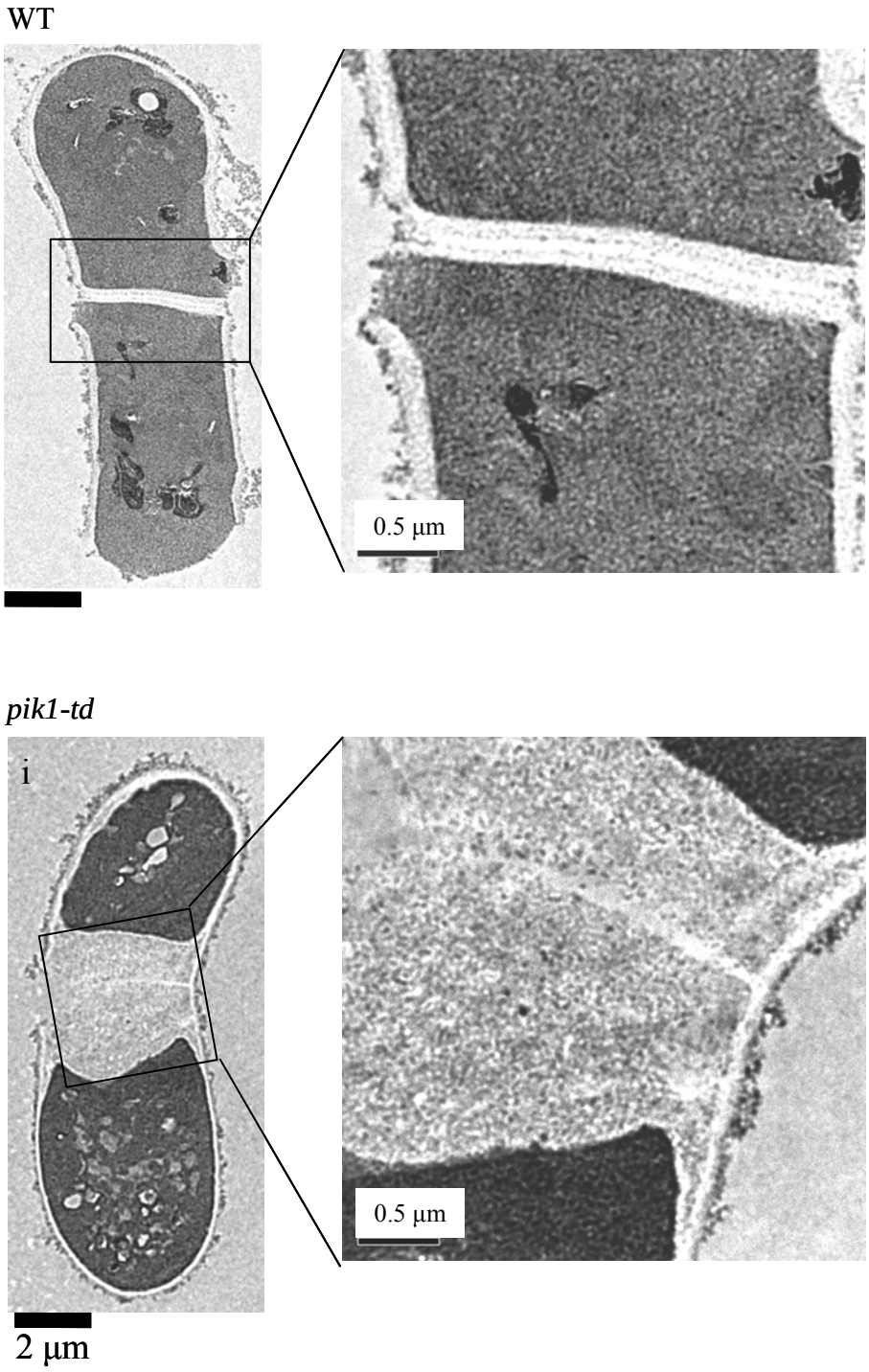


Figure 3.23. (continued)

B. Continued

pik1-td

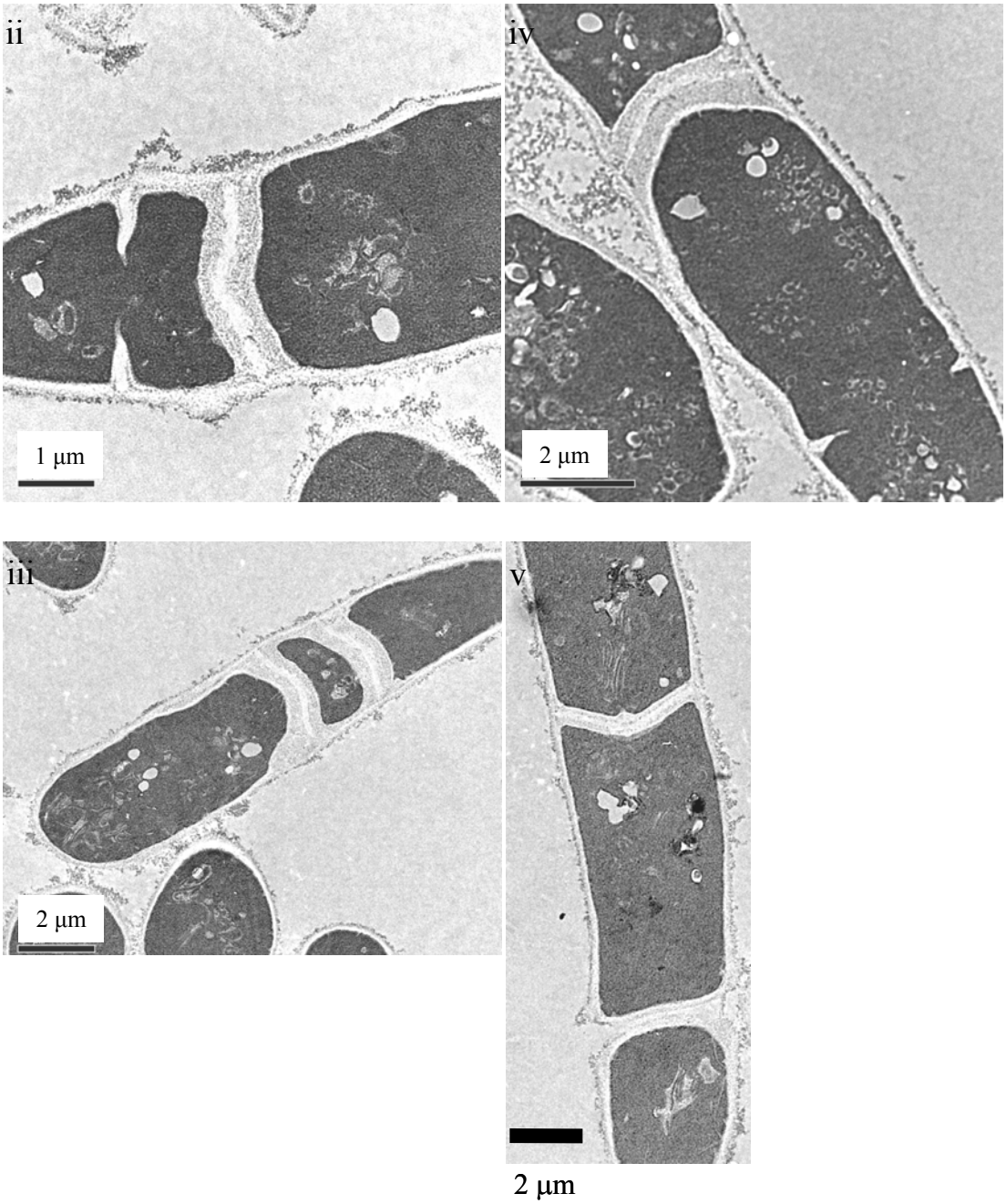


Figure 3.23. (continued)

C

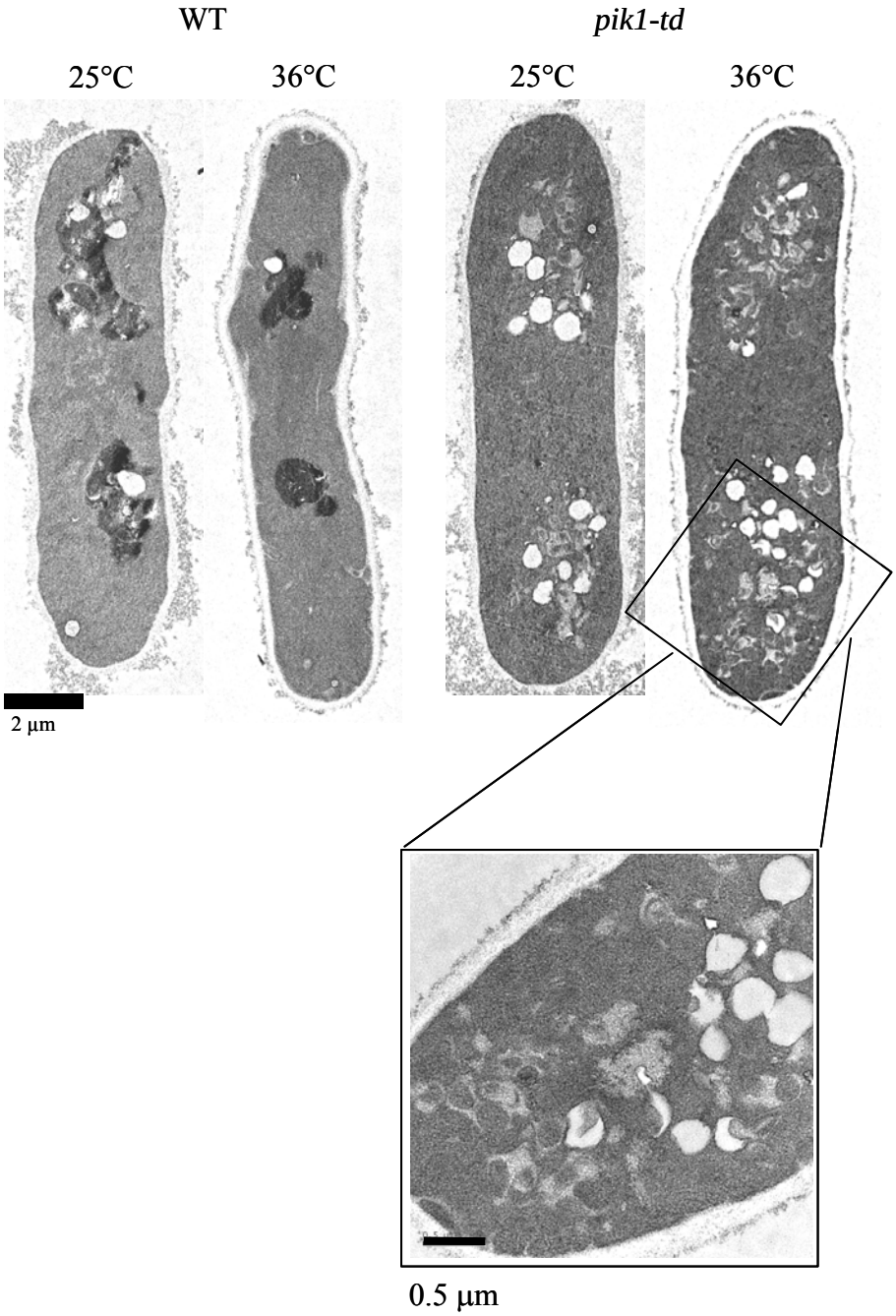


Figure 3.23. (continued)

3.4. *S. pombe* Pik1p is periodically found at the medial plane of cell

It can be expected that the subcellular location where *S. pombe* Pik1p can be visualized might correspond to locations where the protein carries out its functions. To determine the subcellular localization of Pik1p in *S. pombe*, I used an enhanced GFP (eGFP) fusion approach. The study of Pik1p localization would confer understanding regarding the Pik1p function at certain cellular compartment(s) during cell cycle.

3.4.1. Expression of 2XeGFP-*pik1* supports viability and proliferation of cells carrying the Δ*pik1*::*ura4* chromosomal allele

To determine the subcellular localization of Pik1p, I tried to obtain cells with an eGFP coding sequence integrated, in frame 3′ to the *pik1* chromosomal coding sequence. If successful, expression of the fusion gene would be controlled by the native *pik1* promoter, resulting in a C-terminal fusion protein, Pik1p-eGFP. In several independent trials no viable haploid cells were isolated. Therefore, an N-terminal tagging approach was chosen. I first constructed an episome, pREP41-2XeGFP-*pik1*, on which two eGFP coding sequences were fused in frame to the 5′-end of a *pik1* cDNA coding sequence. pREP41-2XeGFP-*pik1* was introduced into hemizygous *pik1*/Δ *pik1*::*ura4* diploid cells (strain N1231). After inducing meiosis and performing random spore analysis, I was able to select haploid cells devoid of a functional chromosomal *pik1* locus (Δ*pik1*::*ura4*) and containing pREP41-2XeGFP-*pik1* as the sole source of Pik1p (strain N1369).

Cell proliferation was determined when cells were cultured in the presence or absence of thiamine. After preculture overnight at 30°C in the presence of thiamine, cultures were started at the same cell density in the presence or absence of thiamine and incubated for up to 72 hours. In both cases, cells proliferated regardless of the presence or absence of thiamine; doubling times were 3.6 hours or 4.0 hours in the repressed or derepressed condition, respectively (Figure 3.24. A, page 150).

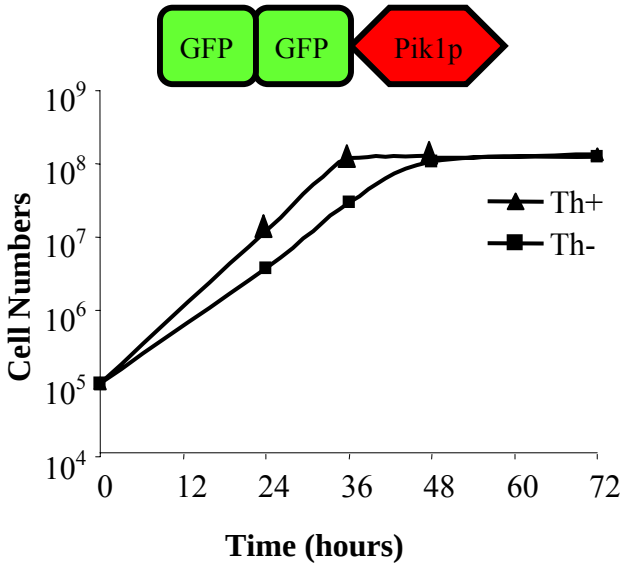
To confirm whether the 2XeGFP-Pik1 fusion protein was stable, I performed western blot analysis (Figure 3.24. B). I used two primary antibodies, anti-GFP and anti-Pik1 to detect the GFP and Pik1p moieties of the fusion protein. Cells cultured with or without thiamine were collected at the mid-logarithmic phase of growth for preparation of total protein extracts. The amount of protein extract loaded was visualized with

Ponceau S solution before antibody reaction. Two blots showed that equal amounts of protein extracts from repressed or derepressed condition were loaded. One blot was used for the anti-GFP reaction and the second for the anti-Pik1 reaction. The predicted size of the 2XeGFP-Pik1 fusion protein was approximately 149 kDa. As shown in Figure 3.24. B, the anti-GFP as well as anti-Pik1 only detected the fusion protein band from the protein extract in the derepressed condition (arrow). The eGFP tag was not cleaved from the fusion protein in these cells. This was confirmed by western blot analysis with total protein extracts of the cells. The anti-GFP did not detected the eGFP tag along.

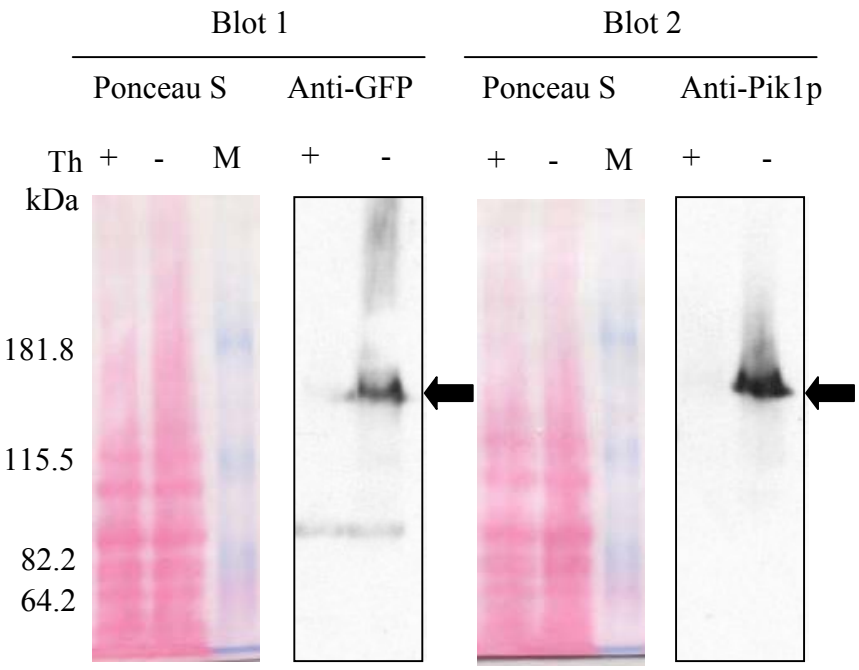
F-actin ring and septum formations were visualized in cells under the repressed or derepressed condition (Figure 3.24. C, page 151). Cells in the mid-logarithmic phase of growth were collected and fixed with formaldehyde. F-actin patches were distributed at one or both tips, and F-actin ring was assembled under both conditions. The proportion of cells with F-actin ring was also similar: 12% under the repressed condition, and 10% under the derepressed condition. The fixed cells were stained with calcofluor white and DAPI for visualizing septum and DNA, respectively. Septum was formed between completely segregated nuclei under the both conditions. The proportion of septating cells was similar: 8% under the repressed or derepressed condition.

These observations indicate that the 2XeGFP-Pik1 fusion protein was stable. The fusion protein expressed under repressed condition was sufficient for cell viability although it was not detected by western blot analysis. Also, the fusion of 2XeGFP to the N-terminal *pik1* did not affect cell growth and division in the absence of the chromosomal *pik1* locus, suggesting that the fusion of 2XeGFP to the N-terminal *pik1* had no apparent effect on Pik1p functions.

A


GFP
GFP
Pik1p
Cell Numbers
10^9
10^8
10^7
10^6
10^5
10^4
0
12
24
36
48
60
72
Th+
Th-
Time (hours)

B


Blot 1
Blot 2
Ponceau S
Anti-GFP
Ponceau S
Anti-Pik1p
Th + - M + - + - M + -
kDa
181.8
115.5
82.2
64.2

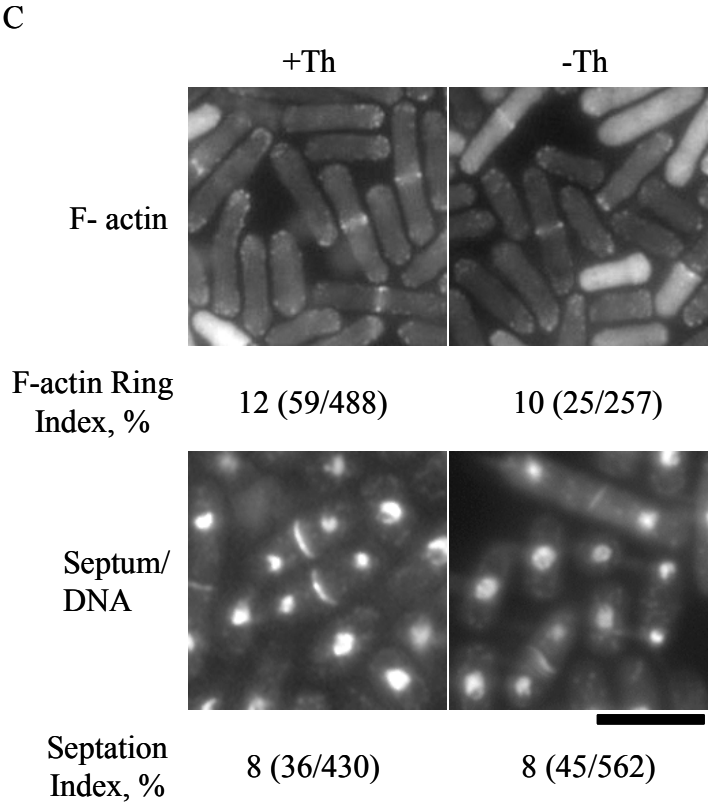


Figure 3.24. 2XeGFP fusion to Pik1p N-terminus does not affect cell cycle control in cells lacking the chromosomal *pik1* coding locus. (A) Cell proliferation in the presence (▲) or absence (■) of thiamine for up to 72 hours. Cultures were started at cell density of 1 x 10^5 cells/mL (time 0 hour) in the presence or absence of thiamine from an overnight pre-culture at 30°C in the presence of thiamine. The cell proliferation graphs are similar sigmoid regardless of repressed or derepressed condition. (B) The 2XeGFP-Pik1 fusion protein accumulates in the cell. Cells cultured in the presence or absence of thiamine at the mid-log were collected for total protein extract. Staining blots with ponceau S solution showed the loading amount of total protein extract. Western blot analysis was performed using two primary antibodies, anti-GFP and anti-Pik1 serum. In both cases, a polypeptide of approximately 149kDa was detected only from the protein extract of cells cultured under the derepressed condition (arrow). '+' indicates the addition of thiamine, and '–' indicates the absence of thiamine. (C) Visualization of F-actin distribution and septum formation. Cultures were the same as described in (A), but cultures only lasted for 24 hours at 30°C. Cells were fixed with formaldehyde. Cells were used for the visualization of F-actin, septum, or DNA with FITC-phalloidin, calcofluor white, or DAPI, respectively. The formation and morphology of F-actin rings/patches and septum formation were normal regardless of the presence or absence of thiamine. F-actin ring index or septation index showed similar cell proportions containing F-actin rings or septa, respectively, in both cells regardless of the presence or absence of thiamine. Numbers in brackets are the number of cells with F-actin ring or septa that were counted. Scale bar, 10 μm.

3.4.2. 2XeGFP-Pik1p is visualized as punctate staining throughout the cytoplasm and as a medial band in cells from asynchronous cultures

I have determined that the 2XeGFP-Pik1p is stable and functional in cells carrying the recombinant chromosomal Δ*pik1*::*ura4* locus (strain N1369). Therefore, it was expected that the 2XeGFP-Pik1 fusion protein would be properly localized in cells for the achievement of *S. pombe* Pik1p functions.

To determine the subcellular localization of the 2XeGFP-Pik1p in cells carrying the recombinant chromosomal Δ*pik1*::*ura4* coding locus, I detected the fluorescent signal of 2XeGFP-Pik1 fusion protein by fluorescence microscopy of cells grown under the derepressed condition (Figure 3.25.). Cells of strain N1369 were precultured in the presence of thiamine overnight at 30˚C. Cultures were started at same cell density in the presence or absence of thiamine, and cells at the mid-logarithmic phase of growth were collected. Live cells were observed. In cells from asynchronous culture under the derepressed condition (-Th), a pattern of punctate dots of fluorescence was observed throughout the cytoplasm and the periphery region. The 2XeGFP-Pik1p fluorescence also accumulated at the medial plane in some cells which still contained the cytoplasmic punctate dot pattern. On the other hand, no fluorescent signal was detected under the repressed condition (+Th).

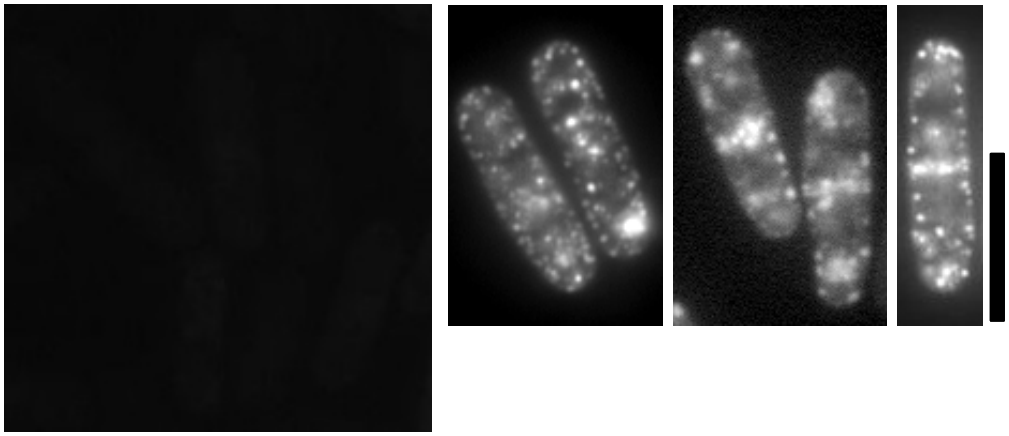


Figure 3.25. Distribution of 2XeGFP-Pik1p in cells from an asynchronous culture. The localization of Pik1p was examined by ectopic expression of a 2XeGFP-Pik1 fusion protein from an episome under the control of an attenuated *nmt*1 promoter in cells lacking the chromosomal *pik1* coding region. Cell cultures were for 20-24 hours at 30°C in the derepressed condition (-Th). Punctate fluorescence was observed throughout the cytoplasm. In some cells, a transverse band of fluorescence was observed at the middle of the cell. On the other hand, no fluorescent signal has been shown under the repressed condition (+Th). Scale bar, 10 μm.

3.4.3. Appearance of Pik1p at the medial plane of the cell corresponds with septum formation in synchronous cultures

Localization of Pik1p at the medial plane of the cell might reflect the observation that Pik1p interacts with Cdc4p, which is a component of the contractile ring that forms also at the medial plane, or our findings that implicate Pik1p in the processes of septation and cell separation. To determine when the medial 2XeGFP-Pik1p fluorescence appears during cell cycle, I performed fluorescence microscopy of cells expressing 2XeGFP-*pik1* after synchronization of haploid *cdc25-22* cells by temperature block and release. At 36°C, *cdc25-22* cells arrest in G2 prior to entering mitosis (Nurse *et al.*, 1976). The effect of expressing a *pik1* cDNA sequence in haploid wild-type *pik1* cells under control of the attenuated *nmt1* promoter on pREP41 plasmid has been investigated previously (Steinbach *et al*., submitted). Cell proliferation was not affected under these conditions in either the presence or absence of exogenous thiamine. It was thus assumed that *cdc25-22* cells expressing the extra episomal 2XeGFP-*pik1* would proliferate properly.

After 4 hours arrest at 36°C, *cdc25-22* cells carrying an episomal 2XeGFP-*pik1* allele were released to 25°C and samples were taken every 20 minutes. Half of each sample was used for fluorescence microscopy, and the remainder was fixed with formaldehyde and stained for visualization of F-actin, DNA, or septa. The degree of cell synchronization achieved was determined by measuring the proportion of cells at each time point that were binucleate, that had an assembled F-actin ring or that were septated. As expected (Balasubramanian *et al*., 2004), each of these indices rose to a peak value and then declined through the cell cycle. The F-actin ring index peaked earliest, followed by the binucleate index and the septation index (Figure 3.26.). The appearance of medial 2XeGFP-Pik1p followed the appearance of septating cells; the peak of the septation index was immediately followed by the peak of the medial 2XeGFP-Pik1p (Figure 3.26). At 80 minutes after release to 25°C, F-actin ring was assembled in around 70% of cell population (Figure 3.26. i). At the same time, cells visualized for septum did not show septum formation yet, nor was 2XeGFP-Pik1p observed at the medial plane. However, the punctate dot pattern throughout the cytoplasm was observed, although the signal was faint. The maximum frequency of septated cells appeared at 100 to 120

minutes, indicating that the F-actin ring assembly precedes the septum formation. The peak frequency of 2XeGFP-Pik1p fluorescence at the medial plane was at 120 minutes. At 120 minutes, F-actin rings were no longer observed. At this time, septa were observed between completely segregated nuclei (Figure 3.26. ii). These results suggest that the timing of the medial Pik1p appearance is consistent with roles for Pik1p in septation and cell separation, supported by our observation that 2XeGFP-Pik1p was found at the medial plane when cells were septating but not when forming F-actin ring.

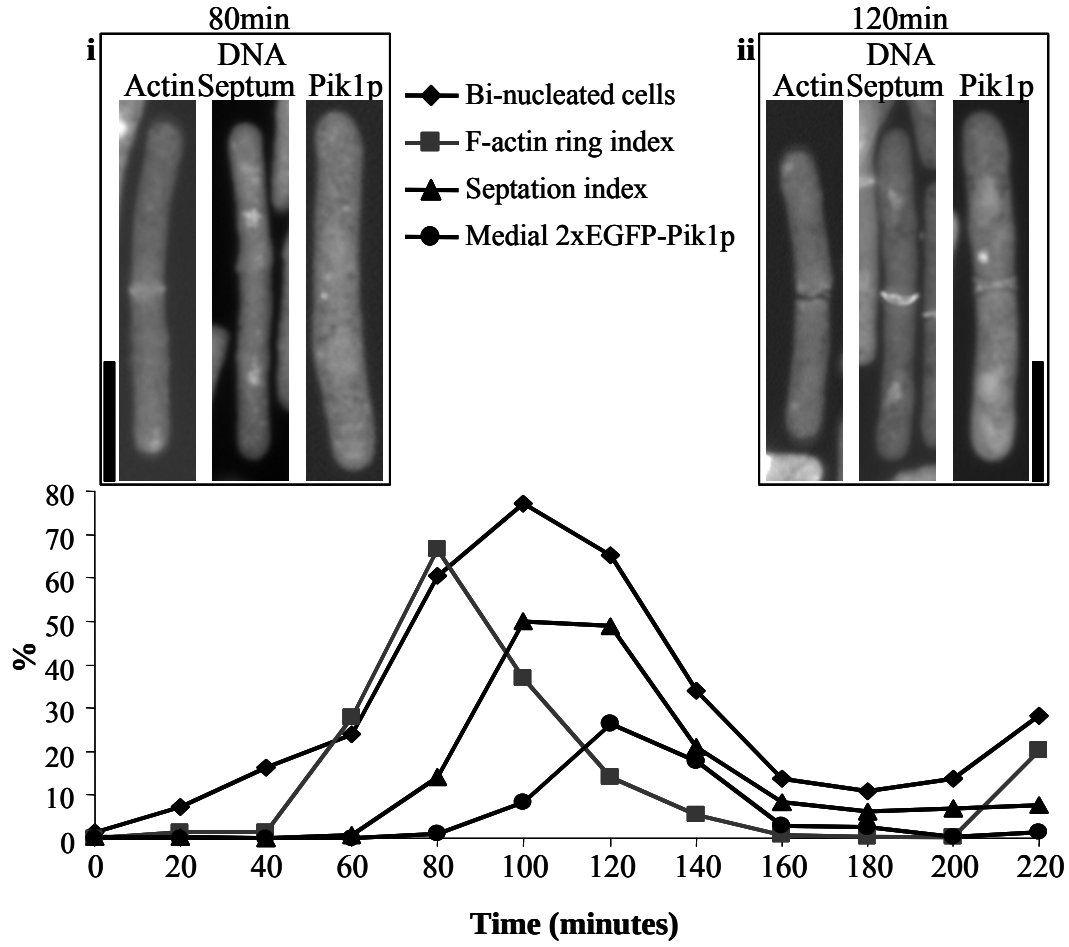


Figure 3.26. Pik1p is periodically localized at the medial plane of the cell. 2XeGFP-Pik1p localization was determined in a synchronous culture. *cdc25-22* cells possessing an episomal 2XeGFP-*pik1*$^{+}$ allele as well as an intact chromosomal locus were cultured at 25°C for 22 - 24 hours and arrested at 36°C for 4 hours. These cells were released to 25°C (time = 0 minute) and two 0.8 mL aliquots of culture were taken every 20 minutes. Cells from a 0.8 mL aliquot of culture were fixed with formaldehyde. Living cells from a parallel sample were observed under a fluorescence microscope. Two trials were performed and the same result was obtained in both trials. At 80 or 120 minutes (i and ii), F-actin or septum/DNA was visualized with FITC-conjugated phalloidin or calcofluor white/DAPI, respectively, in fixed cells whereas 2XeGFP-Pik1p was visualized in live cells. At 80 minutes, F-actin rings were assembled but septa had not formed between nuclei. The punctate 2XeGFP-Pik1p dots are dispersed through the cytoplasm. At 120 minutes, F-actin rings were no longer observed and septa had formed. The medial 2XeGFP-Pik1p band is shown. The graph shows the proportion of cells with 2 nuclei, an F-actin ring, a septum, or a medial 2XeGFP-Pik1p at the times indicated. The peak frequency of observation of the medial 2XeGFP-Pik1p was around 120 minutes. Scale bars, 10 μm.

3.4.4. Colocalization of Pik1p punctate distribution and Gma12-GFP

The punctate and periphery distributions of fluorescently tagged Pik1p was observed in the majority of cells from asynchronous or synchronous cultures. This distribution appeared not to be altered during the cell cycle. Previously, indirect immunofluorescence microscopy was performed with anti-Pik1 serum on methanol-fixed cells that carried a GFP-tagged allele of *gma12*, which encodes a Golgi-associated galactosyltransferase (Figure 3.27, provided by Dr. Desautels). The punctate and periphery immunostaining with anti-Pik1 serum (red) appeared to reflect an association of Pik1p with the Golgi apparatus (yellow-false color), as it was co-localized with GFP-tagged Gma12p color (Green). These punctate and periphery distributions of a Golgi-associated protein on methanol-fixed cells were observed (Ayscough *et al*., 193).

This indicates that *S. pombe pik1* may directly or indirectly participate in Golgi trafficking regardless of cell cycle stages. It is worth noting that the product of the *S. cerevisiae pik1* homologue has been reported to be localized in the Golgi and the nucleus, and plays roles in Golgi trafficking and maintaining Golgi morphology (Walch-Solimena and Novick, 1999; Hama *et al.*, 1999; Audhya *et al.*, 2000; Sciorra *et al.*, 2005; Strahl *et al.*, 2005).

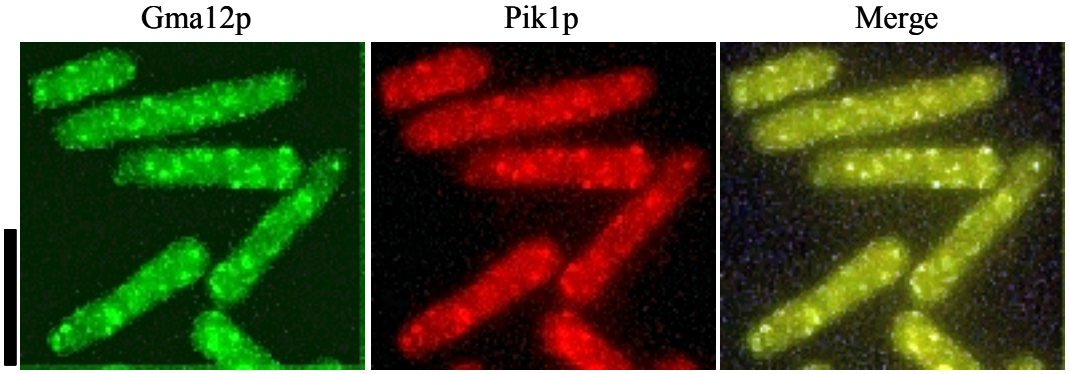


Figure 3.27. Pik1p is a Golgi-associated protein. Cells that carried a GFP-tagged allele of Gma12p (a Golgi-associated galactosyltransferase) were cultured for 24 hours at 30°C, fixed with methanol and processed for indirect immunofluorescence staining with rabbit antiserum against Pik1p and Texas Red conjugated, goat anti-rabbit antibodies. Cells were examined for Gma12p-GFP fusion (green) and Pik1p (red) and the images merged for colocalization (yellow). This experiment was performed by Dr. Desautels.

3.5. Regulation of Pik1p localization and/or activity: search for Pik1p – protein interaction

In *S. pombe*, cytokinesis is regulated by many genes, some of which genetically and/or physically interact each other (Simanis, 2003). *S. pombe* Pik1p was identified as a partner for a myosin essential light chain Cdc4p through a yeast two-hybrid acreen (Desautels *et al.*, 2001). Pik1p homologues in *S. cerevisiae* and mammals have been determined to be localized in the Golgi (Godi *et al.*, 1999; Strahl *et al.*, 2005). A number of proteins identified as interacting with these homologues have also been found in the Golgi (Hendricks *et al.*, 1999; Gavin *et al.*, 2002; Taverna *et al.*, 2002; Haynes *et al.*, 2005). In the following section, I performed a tandem affinity purification (TAP) approach in order to isolate *in vivo* Pik1p-interacting partner(s).

3.5.1. Expression of a TAP-tagged *pik1* allele in haploid cells carrying a *pik1*::*ura4* genomic locus is sufficient for cell viability

TAP tagging is a useful approach to isolate *in vivo* protein-protein complexes. This approach is very attractive because it uses two high affinity purification steps and potentially avoids conditions that might lead to protein denaturation. Moreover, this approach is generally applicable. The same purification protocol can, in principle, be applied to various proteins; whereas, conventional methods must be tailored for each protein. The approach used includes fusing the target protein to 2 protein domains for which affinity ligands are readily available (Figure 3.28., page 161). The first of these is the IgG-binding domain from *Staphylococcus aureus* Protein A (Prot A) which can be purifed on beads that are decorated with IgG molecules. The second is the calmodulin-binding peptide (CBP) which can be purified on beads that are decorated with calmodulin. A site between the Prot A and CBP domains can be cleaved specifically by the Tobacco Etch Virus (TEV) protease. This site is used to release the protein from the first affinity matrix. After the second affinity purification, the target fusion protein is collected in the presence of the calcium chelator, EGTA. After the collection of the target protein, the protein complex can be identified by mass spectrometry.

I used the TAP tagging approach to attempt to isolate *S. pombe* Pik1p-protein complexes. I attempted, but was unable, to isolate haploid cells in which the TAP tag

was integrated, in-frame, at 3′-end of coding region of the genomic *pik1* locus. I subsequently generated an episome in which the TAP tag was fused, in-frame, to the 5′-end of the coding region of a *pik1* cDNA sequence. I refer to this construct as NTAP-*pik1*. The expression of the fusion allele was controlled by an attenuated *nmt1* promoter.

I transformed the hemizygous diploid cells (strain N1231) with pREP41-NTAP-*pik1*. After random sporulation analysis, I selected haploid cells lacking the genomic *pik1* coding region but containing pREP41-NTAP-*pik1* (strain N1240). Figure 3.28. shows a schematic diagram of the use of this strain for purification of protein complexes that contain the NTAP-Pik1 fusion protein.

The proliferation of strain N1240 was similar in the presence or absence of exogenous thiamine. Septum morphology was similar in both cases and appeared to be normal (Figure 3.29., page 162). Thus, as was the case with two other tags that were introduced in the course of this study to the amino terminus of Pik1p, i.e., the 2XeGFP tag and the N-degron tag, the amino terminal TAP tag was innocuous. Since the only *pik1* coding sequence in strain N1240 was the tagged sequence, I conclude that expression of the tagged sequence is sufficient for haploid cell viability. Therefore, the tagged protein is fully functional *in vivo*.

Two preliminary trials for the identification of TAP-Pik1p complexes were performed using mass spectrometry in collaboration with Yeast Resource Center (YRC) in Seattle. Peptides from extracts from strain N1240 were compared to those from extracts from wild-type cells carrying the empty pREP41-NTAP plasmid. Further work will be necessary to optimize the TAP protocol before this work can be carried further.

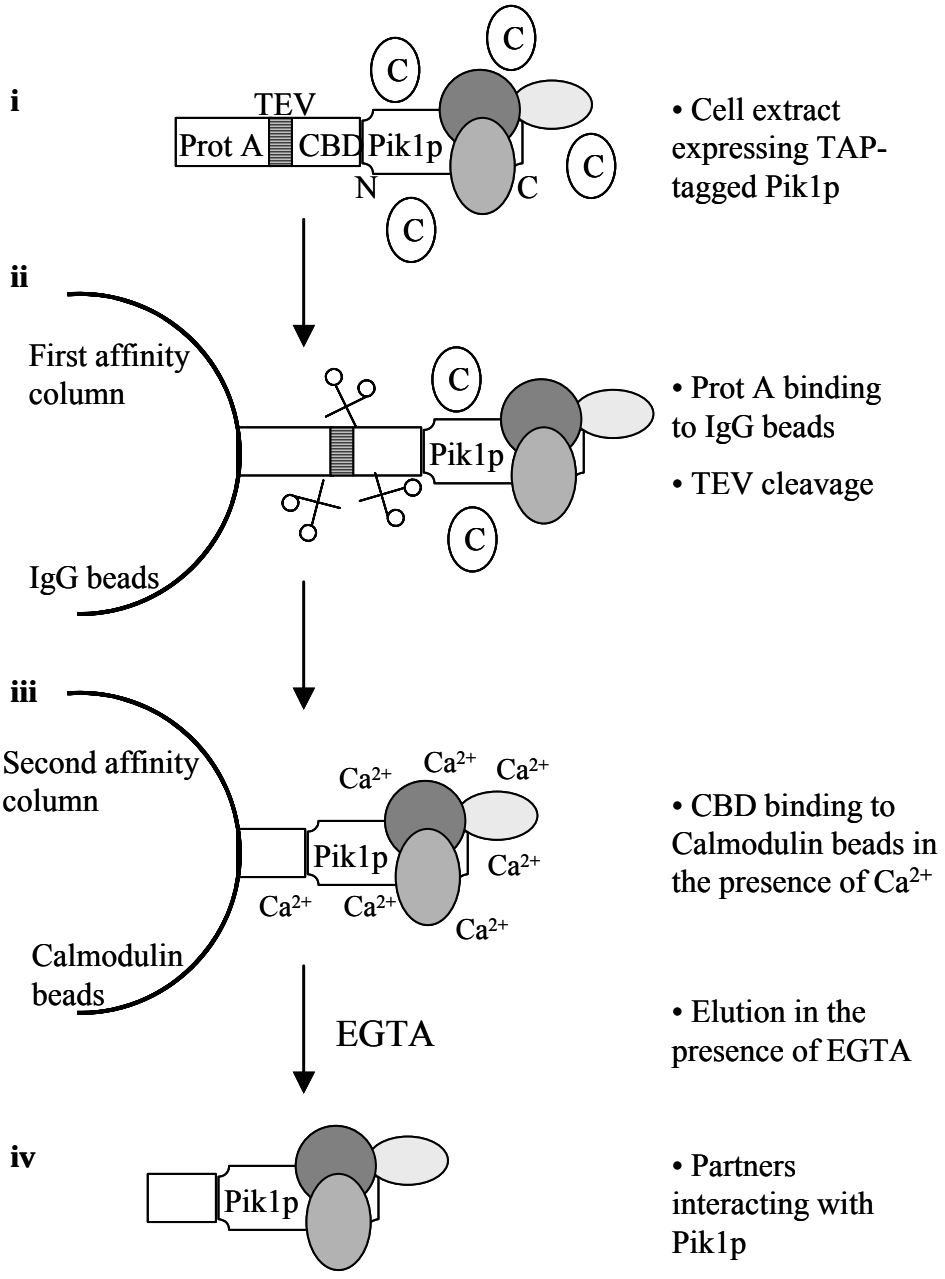


Figure 3.28. Schematic diagram for TAP-Pik1 fusion protein complex purification. (i) Total cell lysate in which TAP-Pik1p interacted with several protein partners (TAP-Pik1 – proteins) and other contaminants (C) coexist (ii) First affinity purification of TAP-Pik1 – proteins and elution using TEV protease (scissors) (iii) Second affinity purification of TAP-Pik1 – proteins in the presence of calcium ions (iv) Second elution using EGTA and purified TAP-Pik1 – proteins complex

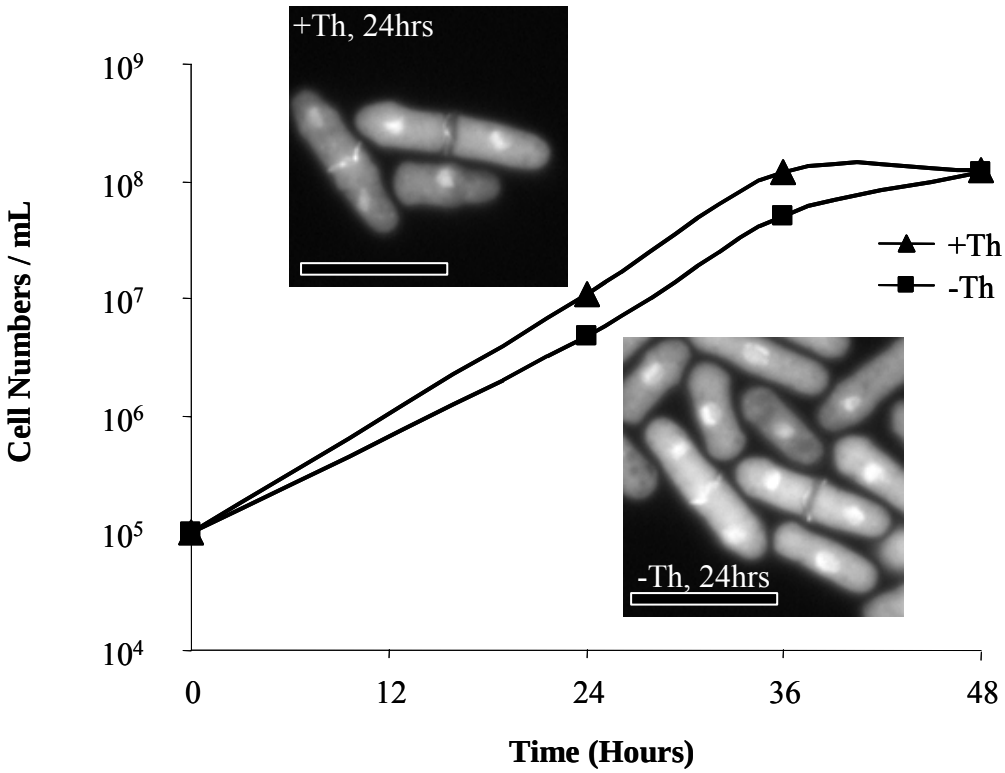


Figure 3.29. Ectopic expression of a TAP-tagged *pik1* in genomic *S. pombe pik1* deleted haploid cells is sufficient for cell viability. Cell proliferation was measured in the presence (▲) or absence (■) of thiamine for up to 48 hours. Cultures started at cell density of 1 x 10^5 cells/mL (time 0 hour) in the presence or absence of thiamine from an overnight pre-culture at 30°C in the presence of thiamine. These cells proliferated and reached saturation with similar cell numbers. Cells at mid-log phase (24 hours) were collected and fixed with formaldehyde. Fixed cells were stained with calcofluor white and DAPI for the visualization of septum and DNA, respectively. Cells were dividing with similar septum morphology between two nuclei regardless of the presence or absence of thiamine. Scale bars, 10 μm.

Chapter 4: Discussion

4.1. Essential functions of *pik1* orthologues are conserved between fission and budding yeasts

Heterologous expression of a gene encoding an eGFP-*S. pombe* Pik1 fusion protein fully complemented the conditionally lethal phenotype of *S. cerevisiae pik1-101*. That is, *S. cerevisiae pik1-101* cells expressing this allele formed colonies with similar efficiencies at both the permissive and restrictive temperatures. It appears that *S. pombe pik1* provides *S. cerevisiae* the functions that are required for colony formation and that are lost to the *pik1-101* allele at the restrictive temperature.

One function known to be lost or diminished in *pik1-101* cells at the restrictive temperature is PtdIns 4-kinase activity (Walch-Solimena and Novick, 1999). The PtdIns 4-kinase activities of Pik1p and Pik1-101p were assayed *in vitro* after immunopurification of the proteins from cells that had been grown at the permissive or restrictive temperatures. The *in vitro* enzymatic activity of the wild-type protein (Pik1p) was much higher from cells grown at the restrictive temperature than from those grown at the permissive temperature. The activity of the mutant protein (Pik1-101p) from cells grown at the permissive temperature was reduced compared to that of the wild-type. The mutant protein from cells grown at the restrictive temperature was even less active. The mutant protein may have some residual enzymatic activity at the restrictive temperature; however, any residual activity would be a small proportion of that produced by the wild-type protein at that temperature.

The Pik1p component of the eGFP-Pik1 fusion protein may be providing PtdIns 4-kinase activity that is required for colony formation and which is missing from *pik1-101* cells at the restrictive temperature. Consistent with this, the wild-type coding sequence but not the kinase-dead allele (*pik1*^{D709A}) fully complemented the conditional

lethality of *pik1-101* at the restrictive temperature. The other *S. pombe* allele tested, eGFP-Pik1^{R838A}, also fully complemented the lethality of *pik1-101* at the restrictive temperature. This allele has been found to have undiminished kinase activity although it may be impaired for some other functions in *S. pombe* (Steinbach *et al*., submitted). The implications of this latter point are discussed in a later section. Thus, 2 alleles of *S. pombe pik1* (eGFP-*pik1* and eGFP-*pik1*^{R838A}) that possess kinase activity fully complement *S. cerevisiae pik1-101* at the restrictive temperature, whereas the kinase-dead allele fails to complement. I conclude that the Pik1p component of the eGFP-Pik1 fusion protein provides the essential PtdIns 4-kinase activity that is lost by the *pik1-101* allele at the restrictive temperature.

Other functions known to be affected in the *pik1-101* mutant at the restrictive temperature are secretion from Golgi to plasma membrane and maintenance of Golgi structures, F-actin distribution, and cytokinesis (Garcia-Bustos *et al.*, 1994; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). *S. cerevisiae* Pik1p is known to be distributed in the Golgi and in the nucleus, and the PtdIns 4-kinase activity is required in both locations for cell viability (Strahl *et al.*, 2005). Since kinase activity is essential in both of these locations and since the *pik1-101* allele loses all or most of its enzymatic activity at the restrictive temperature, the Pik1p component of the eGFP-Pik1 fusion protein may be providing the essential kinase activities in these two compartments. Consistent with this, Pik1p has been found in this study and in a global localization study (Matsuyama *et al.*, 2006), to be a Golgi associated protein in *S. pombe*. Pik1p has not, however, been found to be in the *S. pombe* nucleus. These results are consistent with either of two possible explanations. One possibility is that the Pik1p component of the eGFP-Pik1 fusion protein may be providing the essential kinase activities in the *S. cerevisiae* nucleus. In this case, the Pik1p component may possess signals that direct it to the nucleus. I have searched for nuclear localization and export signal sequences in the Pik1p sequence, without success. It is also formally possible that the eGFP component carries the Pik1p component to the nucleus. If true, this might explain why the eGFP-Pik1p fusion provides complete complementation of *pik1-101* whereas Pik1p on its own provides only very incomplete complementation. If true, we would expect that eGFP possesses signals that allow it to transfer some of the fusion protein to the

nucleus. I have searched for nuclear localization and export signal sequences in the eGFP sequence, without success. If the eGFP component is not responsible for carrying the Pik1p component to the nucleus in *S. cerevisiae*, then I expect that Pik1p may be a nuclear protein in *S. pombe*. The alternative possibility is that the Pik1p component of the eGFP-Pik1 fusion protein does not provide the essential kinase activities in the *S. cerevisiae* nucleus. It may be that Pik1-101p does have residual enzymatic activity and that this activity is sufficient to supply the essential nuclear functions. These two possibilities might be resolved by replacing the chromosomal *PIK1* coding region with *S. pombe pik1* coding region.

4.2. Heterologous expression of *S. pombe pik1* alleles can produce a dominant-negative phenotype in *S. cerevisiae*

Ectopic expression of an *S. pombe pik1* cDNA sequence under the control of the *S. pombe nmt1* promoter slowed the growth of *S. cerevisiae PIK1* cells. This phenotype was not highly penetrant and conditions were found under which *pik1* was able to fully complement *pik1-101* at the restrictive temperature. A similar phenomenon may have been observed when *pik1* was ectopically expressed in *S. pombe*.

In *S. pombe*, a dominant lethal phenotype was observed in two cases in which control of the expression of *pik1* was altered. In one case, a cDNA sequence was under the control of the *nmt1* promoter on a plasmid (pREP1) in *S. pombe* cells that carried the wild-type *pik1* genomic locus (Steinbach *et al*., submitted). In the second case, the genomic promoter of the *S. pombe pik1* locus was replaced with the *nmt1* promoter (Desautels *et al*., unpublished). Changing the regulation of the *pik1* gene might alter the timing of its expression during the cell cycle, or the rate of transcription, or both. The steady state levels of the *pik1* and *nmt1* transcripts during the cell cycle have been studied in a gene profiling study of the genome (Rustici *et al.*, 2004). A cell cycle pattern was not observed for either gene; although, this study would not rule out subtle changes.

In both of the cases described above, the distribution of F-actin was disrupted. For example, contractile ring formation was greatly reduced. This indicates that the altered regulation of the *pik1* gene affects actin cytoskeleton dynamics in *S. pombe*.

Desautels has performed an experiment that may suggest the mechanism for this disruption (Desautels *et al*., unpublished). Desautels found that altered regulation of *pik1* resulted in disruption of the distribution of a recombinant protein that binds to PtdIns(4,5)P_2. PtdIns(4,5)P_2 is produced from PtdIns4P by the activity of a PtdIns4P-5-kinase, and PtdIns4P is the product of the activities of Pik1p and of the other two PtdIns 4-kinases in the cell. Desautels designed a gene encoding the PH domain from a human phospholipase Cδ_1 (PLCδ_1) fused to eGFP (PH-eGFP) as a sensor to be used in living cells to detect the distribution of PtdIns(4,5)P_2. This PH domain is known to bind dominantly to PtdIns(4,5)P_2 (Yagisawa *et al.*, 2006). Desautels introduced the PH-eGFP sensor gene into *S. pombe* cells that were wild-type for the *pik1* gene or into cells in which the promoter of the *pik1* gene had been replaced by the *nmt1* promoter. In the wild-type cells, PH-eGFP fluorescence was observed at the cell tips and at the medial division site. We conclude that concentrations of PtdIns(4,5)P_2 were present at these sites. In cells carrying the mutated promoter, this distribution was disrupted when the *nmt1* promoter that controlled the *pik1* locus was derepressed. The signal of the PH-eGFP fusion protein was no longer localized to the cell tips but was distributed evenly over the cell cortex or throughout the cytoplasm as speckles. It is known that the spatial and temporal regulation of PtdIns(4,5)P_2 is important for actin cytoskeleton dynamics such as actin filament assembly (Sechi and Wehland, 2000). Thus, disregulation of *pik1* in *S. pombe* may disrupt F-actin cytoskeleton dynamics by perturbing phosphoinositide metabolism. A similar mechanism may act in *S. cerevisiae*.

As discussed above, heterologous expression of *S. pombe pik1* under the control of the *nmt1* promoter produced a dominant, negative phenotype in *S. cerevisiae PIK1* cells. In contrast, expression of an eGFP fused *pik1* allele under the control of the attenuated *nmt1* promoter, P_{nmt41}, was apparently innocuous in *S. cerevisiae PIK1* cells. Remarkably, introduction of the kinase-dead mutation, D709A, into this latter construct produced dominant lethality with incomplete penetrance. Homologous expression of kinase-dead alleles of lipid kinases is known in a number of cases to produce a dominant negative phenotype (Schmidt *et al.*, 1996). It is thought that these mutants sequester interacting factors and indirectly inhibit the activity of the endogenous enzyme. This strategy was used as the basis for identification of downstream components by selecting

for suppressors of the dominant negative phenotype. In *S. pombe*, ectopic expression of the kinase-dead allele delayed cell proliferation and increased the septation index. Thus, this ectopic expression may titrate away interacting factors, which would be required for the cell division control. In the case of *S. cerevisiae*, this could also be the case. This may indicate that the PtdIns 4-kinases in both yeasts have conserved interactors with other proteins or metabolites that are involved in cell cycle control.

4.3. Full complementation of *S. cerevisiae pik1-101* was provided by an eGFP-Pik1p fusion but not by Pik1p alone

Under the control of P_{nmt41}, a recombinant sequence encoding an eGFP-Pik1p fusion provided complete complementation of *pik1-101*; whereas, expression of the *pik1* cDNA alone under the control of the same promoter produced incomplete complementation. One conceivable explanation for these observations was proposed in section 4.1. An alternative explanation can be proposed. The *nmt1* promoter provided a greater degree of complementation than the attenuated promoter, and the highly attenuated promoter produced no apparent complementation. Thus, it seems that the incompleteness of the complementation conferred under the control of the *nmt1* promoter resulted from inadequate levels of expression of *pik1*. In addition, cases have been noted previously where GFP tagging has increased the stability of the fusion partner protein (Rucker *et al.*, 2001). So, the complete complementation conferred by the expression of eGFP-*pik1* must be the result of higher levels of expression of the *pik1* component of the fusion. The eGFP-*pik1* fusion may have provided for increased translational efficiency or for greater protein stability compared to the original construct.

I have tried to detect the episomal *S. pombe pik1* expression in *S. cerevisiae* by western blot analysis with polyclonal antiserum against Pik1p or anti-GFP antibody against eGFP. Although the growth of *S. cerevisiae* responded to the episomal *S. pombe pik1* alleles, the gene products were not detected regardless of the presence or absence of thiamine. The level of *S. pombe pik1* expression was sufficient for the effects on *S. cerevisiae* but not for the detection by the western blot analysis.

4.4. Utility of *S. pombe nmt1* promoter and terminator sequences in *S. cerevisiae*

The expression of *S. pombe pik1* under the control of the *nmt1* promoter provided a reproducible, but incomplete, complementation of the conditional lethality of *S. cerevisiae pik1-101* at 37^{o}C. I conclude from this that the *nmt1* promoter sequence is functional to some extent in *S. cerevisiae*. The observation that TATA box mutations that attenuate *nmt1* promoter strength in *S. pombe* weakened the complementation in *S. cerevisiae* suggested that these promoter mutants are also attenuated in *S. cerevisiae*. It also suggests that the incompleteness of the complementation might result from insufficient levels of *S. pombe* Pik1p in *S. cerevisiae*.

The *nmt1* promoter and the mutant, attenuated versions of it, have proven to be useful for molecular genetic studies in *S. pombe*. The *nmt1* gene is involved in thiamine biosynthesis. An *nmt1* null mutant is auxotrophic for thiamine (Maundrell, 1990). The presence of thiamine represses transcription by about 300-fold (Maundrell, 1990; Basi *et al.*, 1993; Forsburg, 1993). In *S. cerevisiae*, the *THI5* gene family is required for thiamine biosynthesis (Wightman and Meacock, 2003). Each member of this gene family (*THI5*, *THI11*, *THI12* and *THI13*) is homologous to the *S. pombe nmt1* gene. Exogenous thiamine represses expression of these genes by about 15-fold in *S. cerevisiae* (Wightman and Meacock, 2003). I have found the *S. pombe nmt1* promoter and the available attenuated alleles of it to be useful for molecular genetic studies in *S. cerevisiae*. *S. pombe* genes that are already cloned in *nmt1* promoter vectors can be conveniently introduced into the *S. cerevisiae* expression plasmid, YEplac181. The level of expression in *S. cerevisiae* can be controlled by use of the attenuated versions of the *nmt1* promoter. If thiamine represses the *S. pombe nmt1* promoter in *S. cerevisiae*, the effect on gene expression must be slight since we observed very similar results with or without the addition of exogenous thiamine.

4.5. *S. pombe pik1* is essential for cell division and probably for spore germination

I determined that *S. pombe pik1* is essential for cell viability by gene deletion in diploid cells followed by tetrad dissection analysis, and by gene deletion in haploid cells and plasmid-loss experiments. This suggests that the essential functions of Pik1p are not redundant to the functions of the other two PtdIns 4-kinases in *S. pombe*. Sequence comparisons of their coding sequences suggest that they are PtdIns 4-kinases. However,

their biological functions have not been determined yet. Nevertheless, the results of my studies indicate that PtdIns4P and/or its derivates produced by these two PtdIns 4-kinases are not sufficient for cell division in the absence of the *S. pombe* Pik1p functions.

It has been reported that the level of the *pik1* transcript peaks in meiosis I (Mata *et al.*, 2002). The regulation of the *pik1* transcript level during the reproductive cycle indicates that its function is required for some biological process. Gene deletion in diploid cells and tetrad dissection indicate that the expression level and/or activity provided from one *pik1* locus in a hemizygous diploid cell is sufficient for meiosis and spore formation. Spore germination seems to require *pik1*; although, some spores germinate and undergo 1 to 2 cell division cycles before arresting. One possible explanation for the latter would be that the presence of Pik1p dispensed from the hemizygous diploid cells during sporulation or the accumulation of phosphoinositides produced through Pik1p activity permits some germination of spores that inherit the deletion allele. In *S. cerevisiae*, the reduction of PtdIns level induced by the loss-of-function of a CDP-diacylglycerol synthase resulted in the failure of spore germination (Shen *et al.*, 1996). Based on the observations in both yeasts, it is suggested that PtdIns and/or its derivates are required for spore germination. Although the molecular mechanism of spore germination in both yeasts is unclear yet, some biological processes can be assumed to be required for spore germination: the secretion pathway in order to deliver new plasma membrane fractions and cell wall materials, and cytoskeleton dynamics in order to maintain polarized growth. Indeed, these biological processes (secretion and cytoskeleton dynamics) are known to require various phosphoinositides (see section 1.3.2.).

4.6. Localization of Pik1p

Results presented in this thesis, including observations made by Desautels (Park *et al*., submitted), indicate that Pik1p is found in the Golgi in *S. pombe*. This conclusion is consistent with observations made in a global localization study (Matsuyama *et al.*, 2006). It is also consistent with observations of orthologous proteins in other organisms (de Graaf *et al.*, 2002; Strahl *et al.*, 2005). Unlike the case in a number of other organisms, I have not found direct evidence for a nuclear pool of Pik1p in *S. pombe*.

Observations discussed in section 4.1. that may be relevant to this question are discussed below. In this thesis *S. pombe* Pik1p was found to be localized at the medial division plane periodically during the cell cycle. This is a novel observation.

Matsuyama *et al*. performed a global analysis of the localization of proteins in *S. pombe* (2006). The only location they observed for Pik1p was in the Golgi. They did not report Pik1p in the nucleus and they did not note its presence in the medial region of the cell as is reported in this thesis. This study was performed by fusing a YFP coding sequence in frame to the 3´- end of genomic open reading frames and then observing individual cells by fluorescence microscopy. To determine specific cytoplasmic localizations, these fusion proteins were colocalized with a number of eGFP fused marker proteins. The strain that was studied to determine the distribution of Pik1p, contained the chromosomal *pik1* locus and an integrated *pik1*-YFP fusion allele under the control of the *nmt1* promoter. This fusion allele encoded a C-terminal fusion protein Pik1-YFP.

I attempted to introduce eGFP into the genome of haploid *S. pombe* cells as a C-terminal tag for *pik1*. However, I was unable to recover cells with the correct insertion event. Similarly, Desautels failed to produce a TAP tagged *pik1* genomic allele. Both of these studies were performed in haploid cells. If C-terminal tagging impaired Pik1p function then cells with such a tag might not survive since *pik1* is essential for cell viability as shown in this thesis. In the case of the study of Matsuyama *et al*., the strain still contained the intact wild-type *pik1* which would be sufficient for cell viability. The extra C-terminal fusion protein may have been impaired with respect to medial localization. Alternatively, the transient appearance of Pik1p at the medial plane of the cell was simply missed.

A similar global protein localization study involving C-terminal fusions was performed previously in *S. cerevisiae* (Huh *et al.*, 2003). It was reported that *S. cerevisiae* Pik1p was localized in the cytosol (Huh *et al.*, 2003) whereas its localization has been determined to be in Golgi and in the nucleus by using an N-terminal fusion protein (Strahl *et al.*, 2005). The N-terminal *S. cerevisiae* Pik1p fusion was stable as well as functional as judged by the restoration of viability to an *S. cerevisiae PIK1*-null mutant (Strahl *et al.*, 2005). The C-terminal *S. cerevisiae* Pik1p

fusion was found to be stable, but not functional (Strahl *et al.*, 2005). I found that the N-terminal *S. pombe* Pik1p fusion restored the viability of an *S. pombe pik1*-null mutant and the defective cell viability of a temperature-sensitive *S. cerevisiae* mutant, *pik1-101*, at a restrictive temperature (Park *et al*., submitted).

The result presented here in combination with the unpublished data of M. Desautels (Figure 3.27., page 183) indicated that *S. pombe* Pik1p is associated with the Golgi. The Golgi-associated Pik1p may be involved in the biological functions of the Golgi. These include regulation of the transportation of factors participating in septation and cell separation, or of retrograde plasma membranes and vesicles. As discussed in the Introduction, phosphoinositides involved in the regulation of Golgi traffic are under the metabolic control of phosphoinositide kinases and phosphatases that are associated with the Golgi complex (De Matteis and Godi, 2004; De Matteis *et al.*, 2005; Di and De, 2006). The phenotype associated with loss-of-function of Pik1p is similar to the defective cell separation phenotype of an exocyst mutant. In the exocyst mutant, *sec8-1*, Golgi-secretory vesicles accumulated in the vicinity of the septum and exocytosis was defective (Wang *et al.*, 2002). Thus, the *S. pombe* exocyst mutant failed to separate daughter cells (Wang *et al.*, 2002). This indicates that in *S. pombe*, the secretion from the Golgi controlled by the exocyst seems be involved in cell separation. Thus, the Golgi-associated Pik1p may regulate the Golgi traffic, which could affect cell separation. This can be addressed by observing whether exocytosis occurs in the absence of the *S. pombe* Pik1p functions.

In *S. pombe* cells that lost *pik1* functions (*pik1-td* cells), inner membranous or vacuole-like structures were observed that were not observed in wild-type cells. Such abnormal structures were also found in the absence of the *S. cerevisiae PIK1* functions (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). It has been suggested that the functions of *S. cerevisiae PIK1* might be involved in the maintenance of Golgi and vacuole membranes (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). Likewise, the mammalian PI4KIIIβ functions in the maintenance of Golgi structures because its kinase-dead mutant failed to maintain the structural integrity of the Golgi complex (Godi *et al.*, 1999). Thus, *S. pombe pik1* is likely involved in Golgi traffic, at least through maintaining Golgi and/or vacuole membrane dynamics.

In *S. cerevisiae*, Pik1p was also found in the nucleus although its biological functions are uncertain (Strahl *et al.*, 2005). However, nuclear *S. pombe* Pik1p was not detected in this study or in the global analysis of the localization of proteins in *S. pombe* (Matsuyama *et al.*, 2006). It might be that the nuclear portion of *S. pombe* Pik1p was not detected with our current detection condition. Alternatively, there might not be a nuclear pool of Pik1p in *S. pombe*. As discussed in section 4.1., one interpretation of the results of my study of the complementation of *S. cerevisiae pik1-101* by *S. pombe pik1* is that some of the protein may be nuclear. Pik1p orthologues in *S. cerevisiae* and mammals are reported to be localized in the nucleus although the functions are uncertain (de Graaf *et al.*, 2002; Strahl *et al.*, 2005). In the case of *S. cerevisiae*, nuclear Pik1p lipid kinase activity is essential (as described in section 1.3.3.2.) (Walch-Solimena and Novick, 1999; Strahl *et al.*, 2005). In addition, the nuclear Pik1p was detected when its expression was under the control of the strong *GAL1* promoter (Walch-Solimena and Novick, 1999), indicating that it was detectable when *S. cerevisiae* Pik1p was abundant inside cells. It may be that only a small fraction of the endogenous *S. cerevisiae* Pik1p is localized in the nucleus.

The localization of Pik1p to the medial plane of the cell that was observed in the studies reported here is a novel finding. However, it is not clear yet what structures the medial Pik1p pool is associated with. Medial Pik1p could be localized in medial Golgi-derived vesicles, in partitioned Golgi, or associated with structures of the cell cortex. It is not known how *S. pombe* partitions the Golgi into daughter cells during cell division. If *S. pombe* Golgi were dynamically generated and partitioned during cell division like during the mammalian cell cycle, it is possible that the medial Pik1p could be localized in partitioned Golgi which is then translocated to the medial plane. Alternatively, if it does not proceed the way mammalian Golgi does, it is possible that the medial Pik1p could be localized in medial Golgi-derived vesicles or Golgi-disassociated Pik1p could be transported to the medial cell cortex. In mammals, many Golgi-derived proteins were identified at the cleavage furrow during cytokinesis (Otegui *et al.*, 2005). In particular, mammalian Nir2, a regulator in membrane traffic and cytoskeletal dynamics, is Golgi-associated and some portion of Nir2, which is disassociated from Golgi, is also found at the cleavage furrow at the time of the cell division (Litvak *et al.*, 2004). Nir2 may be

involved in the regulation of the cleavage furrow ingression (Litvak *et al.*, 2004). By whatever means *S. pombe* Pik1p is localized in the medial plane, the medial localization of *S. pombe* Pik1p may be required for processes in late cytokinesis: the activity of Pik1p might be required at the division site in order to produce PtdIns4P and consequently to regulate septation and cell separation. The localization study of *S. pombe* Pik1p implies that this protein may be involved in different biological processes in its various subcellular localizations.

The timing of the appearance of the medial Pik1p during the cell division cycle was measured using synchronous cultures. Ideally, a synchronized cell population would proceed through each cell division stage in perfect synchrony. However, real synchronized cells are not placed in the exact same cell division stage and they lose synchrony as generations pass. Despite this limitation, this method contributes to some understanding of the timing of events during cell division. Using this method, I observed that the medial *S. pombe* Pik1p appeared at the time of septation. The appearance of the medial Pik1p followed the appearance of the F-actin ring. Its first appearance was even later than the first appearance of septation. Thus, the medial localization of *S. pombe* Pik1p appears to occur in late cytokinesis, probably during septation and cell separation. If so, this observation is consistent with its functions implicated by the phenotypes of the *pik1-td* cells at the restrictive temperature 36°C. In synchronous cultures, the frequency of cells with the medial 2XeGFP-Pik1p distribution peaked at a value of about 30% at 120 minutes after release from the *cdc25-22* block. This peak percentage was lower than those for the other structures observed. It could be that this method of synchrony induction does not produce a high level of synchrony for the process that we are observing. Alternatively, it may be that only the strongest fluorescent signals from the structure that contains the 2XeGFP-Pik1p at the medial plane are detected under the conditions of the experiment. The fluorescent signals from this structure were not very bright.

The localization study of Pik1p implies that the subcellular localization of PtdIns4P may be discrete in *S. pombe* cells. Some studies in various organisms including *S. pombe* suggest that the localizations of phosphoinositide kinases and phosphatases control the discrete subcellular localizations of PtdIns metabolites. *S. pombe* 3-

phosphatase, Ptn1p, and its substrate PtdIns(3,4,5)P_3 are found at the division site (Mitra *et al.*, 2004). Normally, PtdIns(3,4,5)P_3 must be rapidly converted to PtdIns(4,5)P_2 since a medial pool of PtdIns(3,4,5)P_3 is not observed in wild-type cells, but is observed in mutant cells that lack the Ptn1p phosphatase activity that converts PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 (Mitra *et al.*, 2004). In *S. pombe*, PtdIns(4,5)P_2 is found at the division site and the cell tips as well (Desautels *et al*., unpublished). Discrete localization of the PtdIns metabolites has also been reported in *D. discoidium*, *Drosophila*, and mammalian cell lines (Janetopoulos *et al.*, 2005; Wong *et al.*, 2005; Field *et al.*, 2005). In *D. discoidium*, PtdIns(3,4,5)P_3 and PtdIns 3-kinase were found at the cell's leading edge (cell poles) (Janetopoulos *et al.*, 2005). PTEN, *S. pombe* Ptn1p homologue, was found at the cleavage furrow during cytokinesis and chemotaxis (Janetopoulos *et al.*, 2005). In *Drosophila*, PtdIns(4,5)P_2 was present at plasma membrane including cleavage furrow but PtdIns4P was found in vesicular structures near the poles of the cell during cleavage (Wong *et al.*, 2005). In mammalian cell lines, PtdIns(4,5)P_2 was observed at the cleavage furrow during cytokinesis (Field *et al.*, 2005). The regulation of *S. pombe pik1* gene expression does not appear to be achieved predominantly at the level of transcription or translation since transcript levels are constant during the cell cycle (Rustici *et al.*, 2004) and since Pik1p appears to be present throughout the cycle. Rather, Pik1p functions appear to be regulated by the localization of the protein, and and its substrate and product.

4.7. Pik1p is required for cytokinesis

S. pombe Pik1p is localized in the Golgi. It may or may not be present in the nucleus and it is present at the site of division late in cytokinesis. Loss of *pik1* functions did not affect the selection of the division site or the assembly or constriction of the contractile ring. Thus, Pik1p functions do not appear to be required for the early stages of cytokinesis. Loss of Pik1p functions lead to failure of processes in late cytokinesis. For example, secondary septum material was deposited in an unregulated manner. The secondary septum became abnormally thick. Perhaps, a signal for cessation of secondary septum material deposition failed to be made, transmitted or received. Hydrolysis of the primary septum failed to occur. This failure might plausibly be a direct effect of lack of

Pik1p function or an indirect effect. That is, the abnormally thickened secondary septum might be a barrier to hydrolysis of the primary septum. In the absence of Pik1p functions, multiple septa form. Pik1p may act to suppress the reinitiation of septation in a given cell cycle.

As described in section 1.2.2., *S. pombe* cytokinesis is achieved by several steps and by the orchestrated actions of numerous components. Some of the components that are known to regulate septation or cell separation contain pleckstrin homology (PH) domains. PH-domains in many proteins bind phosphoinositides (De Matteis *et al.*, 2005). Mid2p is an *S. pombe* protein that is required for cell separation and which has a PH-domain (Berlin *et al.*, 2003; Tasto *et al.*, 2003; An *et al.*, 2004), although phosphoinositide-binding has not yet been determined. Other proteins that are required for cell separation are also thought to bind phosphoinositides. For example, *S. pombe* has 4 septins that are required for cell separation. Although phosphoinositide-binding by these septins has not been demonstrated in *S. pombe*, it has been demonstrated for homologous proteins in *S. cerevisiae* and in mammals (Zhang *et al.*, 1999; Casamayor and Snyder, 2003). Thus, 2 types of proteins that are required for cell separation, Mid2p and septins, each likely bind phosphoinositides in *S. pombe*. The PH-domain of Mid2p has been shown to be required for recruitment of Mid2p to the site of division (Berlin *et al.*, 2003). It seems likely, given that PH-domains bind to phosphoinositides, that the recruitment of Mid2p to the site of division is regulated by phosphoinositides. Given that the phenotypes of loss of functions of Mid2p, the septins and Pik1p are similar, i.e., failure of cell separation, it seems plausible that Pik1p may regulate the recruitment of Mid2p and the septins to the division site. This regulation would be achieved by the activity of Pik1p at the division site in the late processes of cytokinesis in order to control the accumulation of phosphoinositides. It has been determined that the septins and Mid2p are also localized to the division site in late cytokinesis (Wu *et al.*, 2003; Tasto *et al.*, 2003).

The study of *its3* in *S. pombe* also suggests that the regulation of the accumulation of phosphoinositides at the division site may be required for cytokinesis. Its3p appears to be the only PtdIns4P 5-kinase in *S. pombe* (Wood *et al.*, 2002). Loss of Its3p function was reported to result in increased PtdIns4P levels and decreased

PtdIns(4,5)P_2 levels in total cell extracts (Zhang *et al.*, 2000). When cells lose Its3p functions, the frequency of cells containing septa increases (Zhang *et al.*, 2000). Its3p accumulates at the septation site and is known to regulate cell wall integrity via affecting the *S. pombe* Rgf1/Rho1 pathway (Deng *et al.*, 2005). The mechanism by which the substrate of Its3p, PtdIns4P, is provided at the septation site is uncertain. Based on the phenotypic analyses of loss of functions in Pik1p and Its3p, and their subcellular localizations, it is possible that the medial Pik1p may provide a PtdIns4P pool for Its3p in the late stages of cytokinesis.

Golgi-associated *S. pombe* Pik1p may also be involved in endocytotic processes that are involved in cytokinesis. Endocytosis in *S. pombe* occurred at the region of actively growing tips and at the division site (Gachet and Hyams, 2005). Endocytosis at both sites was found to depend on F-actin, based on studies with Latrunculin B (Gachet and Hyams, 2005). My result showed that disruption of F-actin patch distribution was observed when cells lost the functions of Pik1p. However, the assembly and constriction of the F-actin contractile ring was not affected. Abnormal F-actin distribution was also reported to be associated with loss of function of *pik1* orthologues in *S. cerevisiae* and *Drosophila* (Walch-Solimena and Novick, 1999; Brill *et al.*, 2000). In *S. pombe*, Pik1p is, thus, involved in cytokinesis *via* regulating the distribution of F-actin patches and consequently affecting endocytosis.

In summary, study of the *pik1-td* allele has provided insight into the terminal phenotype of cells that have lost Pik1p functions. Study of the 2XeGFP-*pik1* allele has provided insight into the possible locations where Pik1p may carry out its functions. Taken together, the results from these studies have led me to suggest that Pik1p may provide phosphoinositides at the division site that are required to recruit other key proteins to the division site such as Mid2p and septins where they can perform their functions that are required for completion of cytokinesis. Further, Pik1p may provide PtdIns4P at the division site to Its3p which utilizes this as its substrate and which is required for septation.

4.8. Interaction of Pik1p – Cdc4p in *S. pombe*

Our previous studies (Yeast two-hybrid and Enzyme-Linked Immunosorbent Assay (ELISA)) suggested that Pik1p may interact with Cdc4p in *S. pombe* (Desautels *et al.*, 2001; Steinbach *et al*., submitted). There is a possibility that this interaction may not occur in *S. pombe* cells because there is no direct biochemical evidence of it *in vivo*. Our observations however indicate that Pik1p may at least functionally interact with Cdc4p in *S. pombe*. As described in section 4.2., a dominant lethal phenotype was observed in two cases in which control of the expression of *pik1* was altered. In one case, *pik1* was ectopically expressed in *S. pombe* cells by placing a cDNA sequence encoding its coding region on an expression vector under the control of the *nmt1* promoter (Steinbach *et al*., submitted). The R838 residue proved to be critical for expression of this phenotype. The R838 allele was also found to be critical for interaction of Pik1p with Cdc4p in a yeast two-hybrid assay and ELISA. Interestingly, the R838 residue is not required for *S. pombe* cell viability, based on allele replacement studies in diploid cells and tetrad dissection analysis (Steinbach *et al*., submitted). Also, the ectopic expression of the wild-type *pik1* coding sequence did not induce lethality in *cdc4*^{G107S} cells. It is noteworthy that the *cdc4*^{G107} residue is also required for the interaction with Pik1p *in vitro* (Steinbach *et al*., submitted). Furthermore, ectopic expression of the wild-type *pik1* cDNA in cells with a wild-type *cdc4* genomic locus showed a dramatic increase of lipid kinase activity; whereas, in cells with the *cdc4*^{G107S} allele no increase of lipid kinase activity was observed (Steinbach *et al*., submitted). Thus, these two residues, *pik*1^{R838} and *cdc4*^{G107}, contribute to the lethality induced by ectopic expression of *pik1*; the effects may be direct or indirect.

The functional interaction of *pik1* with *cdc4* could occur *via* the regulation of F-actin dynamics. As demonstrated in section 1.2.2.2., F-actin and Cdc4p are required for assembly and constriction of the actomyosin ring. Ectopic expression of *S. pombe pik1* increased the level of mono- phosphoinositides and also disrupted F-actin distribution: the F-actin ring index dramatically decreased and F-actin patches were no longer polarized (Steinbach *et al*., submitted). It is known that phosphoinositides, especially PtdIns(4,5)P_2, regulate actin cytoskeleton dynamics (Sechi and Wehland, 2000); several actin-binding proteins associate with PtdIns(4,5)P_2 and the activities of some actin-binding proteins are regulated by PtdIns(4,5)P_2. The association of actin-binding

proteins with PtdIns(4,5)P_2 and the alteration of their activities result in actin filament assembly or plasma membrane – actin cytoskeleton interactions. It can be assumed that the alterations of phosphoinositides by the action of kinases and phosphatases may consequently affect the regulation of the actin cytoskeleton. Thus, altered *pik1* expression may directly and indirectly regulate the F-actin structures through discrete accumulation of phosphoinositides or unidentified mechanism in order to consequently be involved in cytokinesis.

If Pik1p physically interacts with Cdc4p in *S. pombe* cells, where and when does this occur, and what would be the function of the interaction? We have not found a time and a place when Pik1p and Cdc4p are colocalized. The interaction, if it occurs, may be brief, Pik1p associated with Cdc4p may transiently stay at the medial division site, or this interaction may occur in different subcellular compartments such as the Golgi. Also, only small proportion of each protein pool may be involved in this interaction.

It has been reported that the transcription of *cdc4* is regulated by the transcription factor, Ace2p (Rustici *et al.*, 2004). It is interesting to note that the target genes of Ace2p are mainly ones which encode products that are involved in cell separation (Petit *et al.*, 2005). The yeast two-hybrid screen that identified Pik1p as an interaction partner of Cdc4p also identified Vps27p as a partner for Cdc4p (Desautels *et al.*, 2001). The homologue of Vps27p in *S. cerevisiae* is known to be involved in vesicle traffic (Piper *et al.*, 1995). Vesicle traffic associated with exocytosis and endocytosis is important for cytokinesis in *S. pombe* (Wang *et al.*, 2002; Gachet and Hyams, 2005; Martin-Cuadrado *et al.*, 2005). In *S. pombe*, the exocyst regulates exocytosis and is involved in cell separation (Wang *et al.*, 2002). It may be that in interaction between Pik1p and Cdc4p occurs in association with the Golgi with the processes of exocytosis and encodytosis. Thus, instead of looking at the contractile ring for evidence of this interaction, we may need to investigate the processes of septation and cell separation more closely.

4.9. Future studies

In this dissertation, I have determined that *S. pombe* Pik1p functions in cell division control, especially during cytokinesis. *S. pombe pik1* regulates septation and cell separation. It also seems to be involved in the maintenance of Golgi and vesicle

membranes. Since *S. pombe pik1* is involved in septation and cell separation, it is worth assessing how *pik1* is genetically and/or physically related to the SIN elements. *S. pombe pik1* could be involved in the SIN pathway because abnormal septum initiation and septum material deposition were observed when *pik1* function was lost. Thus, it would be informative to determine how the functions of *pik1* are related to the actions of SIN negative regulators (Cdc16p or Byr4p) or of SIN positive regulators (Spg1p, cdc7p, Sid1p, Sid2p or others). For these purposes, conditional double mutant alleles of *pik1* and each of SIN regulators would be generated and characterized. Cell proliferation and microscopic analyses would be performed. In addition, it will be interesting to observe the relationship of Pik1p and Sid2p because Sid2p is the only SIN component that is localized at the division site during septation. It is not clear yet how Sid2p is translocated to the division site. To observe whether the localization of Sid2p at the division site is affected by the loss of Pik1p functions, an eGFP tagged Sid2p in *pik1-td* cells would be observed at the permissive and restrictive temperatures.

As described in section 1.2.2.5., cell separation in *S. pombe* is achieved *via* the actions of a series of proteins which are involved in exocytosis, the structure of the septum scaffold, and hydrolysis of the primary septum. Any loss-of-function of the septum scaffold proteins, Mid2p and septins, disrupts the ring structure of hydrolyzing enzymes, Agn1p and Eng1p, at the division site. The significance of the ring structure of Mid2p, septins, and the hydrolyzing enzymes is not clear yet, except that this ring structure is required for cell separation. The defect in cell separation was also observed in cells lacking the functions of Pik1p. Cells with loss of the Pik1p functions, however, distinctively showed the phenotype of thickened septum formation. It will be worthwhile to determine if the thickened septum phenotype of cells lacking Pik1p functions is displayed when the functions of Mid2p or the septins are missing. For this determination, a deletion of the *mid2* or *spn1*, *spn2*, *spn3*, or *spn4* coding regions would be generated in *pik1-td* cells. Each double mutant at the permissive and restrictive temperatures would be examined by transmission electron microscopy. This might answer why the ring structure of these structural proteins (Mid2p and septins) is important for cell separation.

Whether Pik1p is involved in the translocation of the hydrolyzing enzymes to the division site is not certain at this moment. To determine this, whether the localization of

the enzymes is affected in the absence of the Pik1p functions should be addressed. Also, whether exocytosis occurs in the absence of Pik1p functions should be assessed because the action of exocytosis might regulate the delivery of the primary or secondary septum materials, as well as the hydrolyzing enzymes to the division site. In addition, whether endocytosis at the division site is affected by the activity of Pik1p should be determined because the action of endocytosis at the division site could regulate the accumulation of the septum material.

The study of whether the regulation of discrete phosphoinositide pools, especially PtdIns4P at the division site, is required for cell separation is also important because it will shed light on phosphoinositides functions during cytokinesis. This could be determined, for instance, by using an eGFP fused PH-domain in a time-course experiment, which might give some information regarding the alteration of the distribution of phosphoinositides. In this case, a PH-domain would be from the four-phosphate-adaptor proteins (FAPP1 and FAPP2), which specifically associate with PtdIns4P. These proteins control Golgi-to-cell-surface membrane traffic in mammals (Godi *et al.*, 2004). Alternatively, a PH-domain in *S. cerevisiae* oxysterol binding proteins (OSBPs) could be used. In *S. cerevisiae*, the biological functions of these proteins are not known. However, the PH-domains from these proteins are known to be predominantly targeted to Golgi and to bind only PtdIns4P and PtdIns(4,5)P_2 (Levine and Munro, 2002; Roy and Levine, 2004). Whether Pik1p produces the substrate for Its3p at the division site should be determined.

Based on the results of the gene knockout and *pik1-td* cell studies, *S. pombe pik1* is essential for cell viability. The lethality of haploid cells carrying the *pik1* deletion allele was rescued by the expression of *pik1* cDNA from the episome, pREP81. The episome contains a highly attenuated *nmt1* promoter. These *pik1*-deletion haploid cells were viable even when the episome was repressed by addition of exogenous thiamine. This suggests that even very low level of leaky expression of *pik1* from pREP81 in the presence of thiamine is sufficient for cell viability. The complete repression of episomal *pik1* expression in these cells should result in lethality. McQuire *et al*. reported that two transcription factors, Thi1p and Thi5p, regulate the *nmt1* promoter in *S. pombe* (2006). Deletion of one of either factors reduced expression from the *nmt1* promoter and

deletion of both factors eliminated expression. Thus, future experimental control of the activity of the *nmt1* promoter might be achieved by deletion of either *thi1* or *thi5*. Repression of episomal *pik1* expression in these strain might be expected to produce a lethal phenotype.

This dissertation suggests that phosphoinositide kinases and/or their PtdIns metabolites are involved in cell division control, especially during cytokinesis. Further investigations such as the identification of the genetic interactions with SIN components and cell separation regulators, and determination of the distribution of PtdIns4P during cell division have to be carried out. These studies using *S. pombe* as a model organism will contribute to understanding the underlying mechanisms of eukaryotic cytokinesis, especially the roles of phosphoinositide kinases and phosphoinositides in the late processes of cytokinesis.

Chapter 5: References

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